

THE UNIVERSITY OF HULL

The Characterisation of Calpain-like Proteins in *Trypanosoma brucei*

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By

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Table of contents

Acknowledgements.....	1
Publication	2
Declaration.....	2
Abbreviations.....	3
Abstract.....	5
1. General Introduction	6
1.1 The calpain protein family	7
1.1.1 The conventional calpains: μ -calpain & m-calpain.....	7
1.1.2 Other members of the calpain family	11
1.1.2.1 Calpain-like molecules in mammals	13
1.1.2.2 Calpain-like molecules in <i>Drosophila</i>	14
1.1.2.3 Calpain-like molecules in <i>Caenorhabditis elegans</i>	15
1.1.2.4 Calpain-like molecules in <i>Trypanosoma brucei</i>	16
1.1.3 The multifunctional enzyme family	21
2. Aims.....	23
3. Materials and Methods.....	24
3.1 Cell culture condition.....	24
3.2 Expression constructs and parasite transfection.....	24
3.3 RNA extraction and qRT-PCR	25
3.4 Protein immunoblotting	26
3.5 Hypotonic/Sonication lysis & detergent extraction analysis.....	26
3.6 Immunofluorescence microscopy	27
3.7 Acyl-biotin exchange assay.....	28
4. Analysis mRNA expression levels of calpain-like proteins in procyclic and bloodstream trypanosomes.....	31
4.1 Introduction.....	31
4.1.1 Control of gene expression in <i>T.brucei</i>	31
4.2 Results.....	33
4.2.1 Calpain gene expression in bloodstream and procyclic forms	33
4.2.2 TbCALP1.3 equally expressed in procyclic, slender and stumpy forms.....	34
4.3 Discussion	37
5. Localization and membrane association of calpain-like proteins	40
5.1 Introduction:.....	40
5.2 Results:.....	41
5.2.1 Membrane association of calpains	41
5.2.2 Cellular localisation of calpain-like proteins.....	45
5.2.3 A C-terminal signal sequence for CALP1.3 flagellar localization	50
5.3 Discussion	52
6. The role of N-terminal myristoylation & palmitoylation in flagellar membrane protein trafficking	55
6.1 Introduction.....	55

6.1.1 The flagellum of <i>T.brucei</i>	55
6.1.2 The cytoskeleton of <i>T.brucei</i> flagellum.....	56
6.1.3 The flagellar membrane and matrix	58
6.1.3.1 Intraflagellar transport(IFT).....	58
6.1.3.2 Lipid rafts in the flagellar membrane.....	59
6.1.3.3 Palmitoylation-mediated targeting.....	60
6.2 Results.....	63
6.2.1 CALP1.3 & SKCRP1.5 are palmitoylated <i>in vivo</i>	63
6.2.2 The N-terminal acylation motif is required for flagellar targeting	65
6.2.3 N-terminal palmitoylation is not the only flagellar targeting determinant	67
6.3 Discussion	72
6.3.1 Dual acylation is required for crossing the selective barrier of the flagellum.....	72
6.3.2 Dual acylation is an important early step in flagellar protein targeting	75
6.3.3 A model for the delivery of flagellar/ciliary membrane proteins	77
6.3.4 A model for the flagellar targeting of dually acylated proteins in <i>T.brucei</i>	78
6.3.5 The flagellar pocket is a highly dynamic organelle for protein trafficking	81
7. Concluding remarks	83
8. Reference	85
9. Appendix	101

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Declaration

I hereby declare that the thesis entitled “The Characterisation of Calpain-like Proteins in *Trypanosoma brucei*” has not been submitted for a degree, diploma or any other qualification at any other university.

Wen Liu

Abbreviations

2D-DIGE	Two-dimensional differential in-gel electrophoresis
ADK	adenylate kinase
ARE	AU-rich element
CAP	Cytoskeleton associated protein
CALP	Calpain-like protein
CM precipitation	Chloroform-methanol precipitation
CNS	Central nervous system
COX	Cytochrome oxidase
DAPI	4-, 6-diamino-2-phenylindole
FAZ	<i>Flagellum attachment zone</i>
FCS	Fluorescence-correlation spectroscopy
FITC	Fluorescein isothiocyanate
FLA 1	<i>Flagellum adhesion glycoprotein 1</i>
FP	Flagellar pocket
FRET	Fluorescence-resonance energy transfer
GFP	Green fluorescence protein
GPCR	G-protein coupled receptors
hPAR 1	human proteinase-activated receptor 1
HPDP-Biotin	N-[6-(Biotinamido)hexyl]-3'-(2'-pyridyldithio)-propionamide
IFT	<i>Intraflagellar transport</i>

NEM	N- Ethylmaleimide
PAT	Palmitoyl transferase
PBS	Phosphate buffer saline
PDE	<i>Phosphodiesterase</i>
PFR	Paraflagellar rod
PGKB	Phosphoglycerate kinase B
qRT-PCR	quantitative Real-Time PCR
RNAi	RNA interference
SDM-79	Semi-defined medium 79
SDS	Sodium dodecyl sulfate
SKCRP	Small kinetoplastids calpain-related protein
Tet	Tetracycline
UTR	Untranslated region
VSG	Variable surface glycoprotein
ZPFM	Zimmerman postfusion medium

Abstract

Calpains are a ubiquitous family of calcium-dependent cysteine proteases involved in a wide range of cell regulatory and differentiation processes. In many protozoan organisms, atypical calpains have been discovered that lack the characteristic calcium-binding penta-EF-hand motif of typical vertebrate calpains and most of these novel calpain-like proteins are non-enzymatic homologues of typical calpains. The gene family is particularly expanded in ciliates and kinetoplastids, comprising 25 members in the parasite *Trypanosoma brucei*. Unique to kinetoplastids, some calpain-like proteins contain N-terminal dual myristoylation/palmitoylation signals, a protein modification involved in protein-membrane associations. We analysed the expression of calpain-like proteins in the insect (procyclic) and bloodstream-stage of *T. brucei* using quantitative real time PCR and identified the differential expression of some of the calpain genes. We also present a comprehensive analysis of the subcellular localisation of selected members of this protein family in trypanosomes. Here, of particular interest is the role of protein acylation for targeting to the flagellum. We show that, although acylation is important for flagellar targeting, additional signals are required to specify the precise subcellular localisation.

1. General Introduction

Proteases catalyse the cleavage of amide linkages in proteins and polypeptides. They have been identified in all the organisms from viruses to vertebrates and are extremely important for biological systems. Without protease, it would take hundreds of years to hydrolyse a peptide bond, whereas a protease can sufficiently degrade approximately one million peptide bonds per second. Proteases have been divided into groups based on the catalytic mechanism used during the hydrolytic process (Sajid and McKerrow, 2002). The main catalytic types are serine, threonine, aspartate, metallo and cysteine proteases, other types of proteases may also exist. This project is derived from an interest of parasite cysteine proteases. Many parasite proteases are considered to be promising chemotherapeutic or vaccine targets. Cysteine proteases have attracted particular attentions because they are critical to the life cycle or pathogenicity of many parasites (McKerrow et al., 1999, McKerrow, 1999, Selzer et al., 1999). The first cysteine protease was purified and characterised from the papaya fruit, *Carica papaya*, and was therefore named papain (Martin, 1885b, Martin, 1885a). Numerous cysteine proteases have been subsequently identified. The cysteine proteases of organisms are now divided into four groups referred to as clans, CA (papain and papain-like), CB (viral proteases), CC (viral protease) and CD (family C13; legumain-like) (Sajid and McKerrow, 2002). The Clan CA consists of the majority of parasite cysteine proteases, and is further divided into several subfamilies. Important parasite proteases are located to family C1 (cathepsin B and cathepsin L-like) and family C2 (calpain and calpain-like). Since members in the C2 family appear to have narrow substrate specificities and are widely involved in cellular regulatory processes (Cuerrier et al., 2005, Goll et al., 2003),

therefore the calpain and calpain-like proteins are of particular interests.

1.1 The calpain protein family

1.1.1 The conventional calpains: μ -calpain & m-calpain

Conventional calpains constitute a large number of calcium-dependent cysteine proteases that are extensively studied in mammalian systems (Goll et al., 2003, Croall and Ersfeld, 2007). The term μ -calpain and m-calpain was first used in 1989 referring to the micromolar Ca^{2+} -requiring and millimolar Ca^{2+} -requiring proteases, respectively (Cong et al., 1989). They are present in all vertebrate cells that have been examined including human, monkey, mouse, rat, bovine, porcine, rabbit and chicken (Goll et al., 2003). Both μ - and m- calpains from vertebrate species are well conserved heterodimeric proteins, consisting of two subunits (Fig.1). The large subunit is typically divided into four domains: a short domain I of unknown function; a domain II harboring the active centre of the enzyme including the key catalytic triad of cysteine, histidine and asparagine (C-H-N motif); a linker domain III and a calcium-binding domain IV containing five penta-EF-hand-type motifs (Strobl et al., 2000). The 28kD small subunit has two domains: a glycine-rich domain V and a domain VI similar to domain IV of the large subunit.

The properties of μ - and m-calpains have been extensively studied. Both calpains are Ca^{2+} -dependent cysteine proteases with a pH optimum of 7.2-8.2. The large subunit has a molecular mass near 80kDa, with the μ -calpain large subunit slightly larger than the m-calpain large subunit (Aoki et al., 1986, Imajoh et al., 1988). The small subunit has a molecular mass of about 28 kDa (Sakihama et al., 1985, Emori et al., 1986).

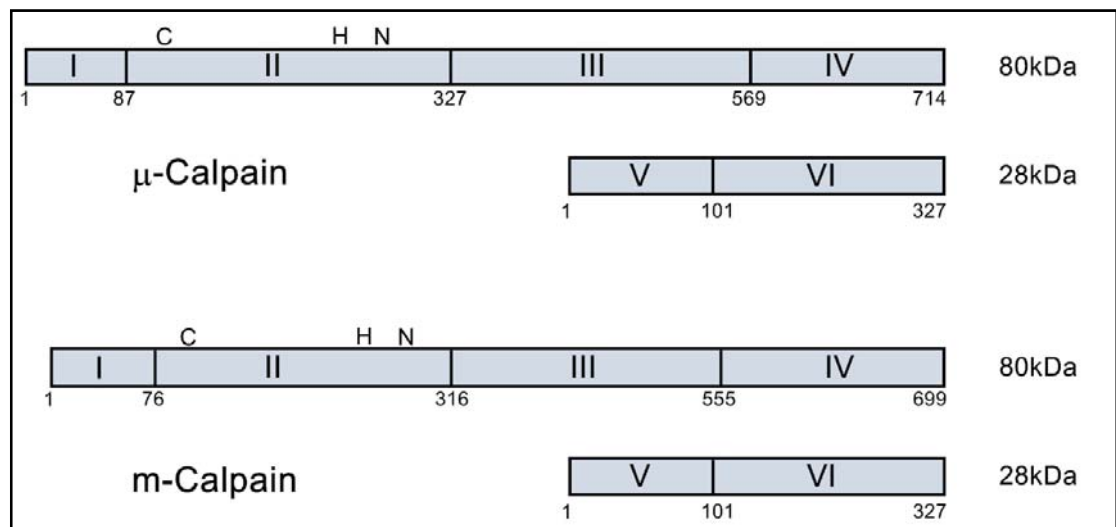


Fig.1 Schematic diagram showing the domain structure of human μ - and m-calpains. Both μ - and m-calpain contain four domain structures and a C-H-N catalytic triad in domain II (Modified from (Goll et al., 2003))

The 28kDa subunit is identical in the μ - and m-calpain molecules and is encoded by a single gene on chromosome 19 in humans (Ohno et al., 1990). The N-terminal region of this subunit, domain V, is glycine (Gly)-rich; 40 of the first 64 residues are Gly (all amino acid positions in this section are for the human calpains unless stated otherwise). There are two Gly stretches in this domain from residues 10 to 20 and from residue 37 to 56. In addition to the Gly-rich region, of the first 101 residues in domain V, 30 are hydrophobic, 26 are polar or charged, a region from residues 78-83 contains five Pro residues (PEPPPP) and a region between residues 91-97 contains four Glu residues (EANESEE). It has been suggested that the hydrophobicity of this domain is involved in phospholipids binding (Imajoh et al., 1986). However, the unordered N-terminal sequence may also imply alternative, yet unknown, uses. The C-terminal part of the 28kDa subunit, domain VI, is a calmodulin-like domain, because the sequence contains four EF hand Ca^{2+} -binding motifs (Ohno et al., 1986) and is marginally homologous to

that of calmodulin (23% identity and 30% similarity). However, subsequent crystallographic studies of this subunit have revealed a fifth Ca^{2+} -binding site (Blanchard et al., 1997, Lin et al., 1997). Therefore, it is also identified as members of the penta-EF-hand family (Xie et al., 2001, Maki et al., 1997).

In addition to the well-characterised small calpain subunit, a gene sharing 63% identity with the 28kDa small subunit has been identified in both human and mice (Schad et al., 2002). There is no Gly stretch in the N-terminal region and the encoded protein binds only weakly to the large 80kDa m-calpain subunit *in vitro* (Schad et al., 2002). Co-expression of this 248 amino acid polypeptide with the 80kDa subunit of m-calpains produces a proteolytically active enzyme having approximately 70% of the activity of m-calpain (Schad et al., 2002). However, disruption of the canonical 28kDa small subunit is embryonically lethal in mice, even though the 248-amino acid polypeptide is expressed simultaneously. Hence, it cannot substitute for the 28kDa subunit in cells and its function is still unclear (Schad et al., 2002).

The 80kDa subunit of μ - and m-calpains are different gene products on chromosomes 11 and 1, respectively (Ohno et al., 1990), but share 55-65% sequence homology (Suzuki et al., 1990). The large subunit of μ - and m-calpains is typically divided into four domains based on their amino acid sequence (Fig.1).

Domain I. The N-terminal domain has no sequence homology with any known sequences; the domain I sequence homology among different species is 72-86%

Domain II. The domain contains C-H-N motif that forms a catalytic triad characteristic of cysteine proteases such as papain. The C-H-N residues are at position 115, 272 and 296 in μ -calpain, and position 105, 262 and 286 in m-calpains. The sequence of domain

II, however, shares little homology with other cysteine proteases, and is probably evolved from a different ancestor. Domain II is highly conserved in different species, with sequence homology ranging from 85% to 93% (Goll et al., 2003).

Domain III. There is no sequence homology of this domain with other proteins. It has been suggested domain III may be involved in binding phospholipids (Tompas et al., 2001) and in regulating calpain activity (Hosfield et al., 1999, Strobl et al., 2000). Sequence analysis indicates two potential EF-hand Ca^{2+} -binding regions, one at domain II/III boundary (329-341 of μ -calpain; 318-338 of m-calpain), and one at the domain III/IV boundary (554-565 of μ -calpain; 541-552 of m-calpain). The Ca^{2+} binding abilities of these regions are, however, not entirely clear, although it has been demonstrated the domain II/III boundary does not have an EF-hand conformation in the crystallographic structure of rat or human m-calpain (Reverter et al., 2001).

Domain IV. Similar to domain VI of the small subunit, the sequence of domain IV is marginally homologous to calmodulin (24-44% identity and 51-54% similarity for μ - or m-calpain), and contains five EF-hand Ca^{2+} -binding sites, with the fifth EF-hand involved in dimerization of the small and large subunits (Fig.2) (Hosfield et al., 1999).

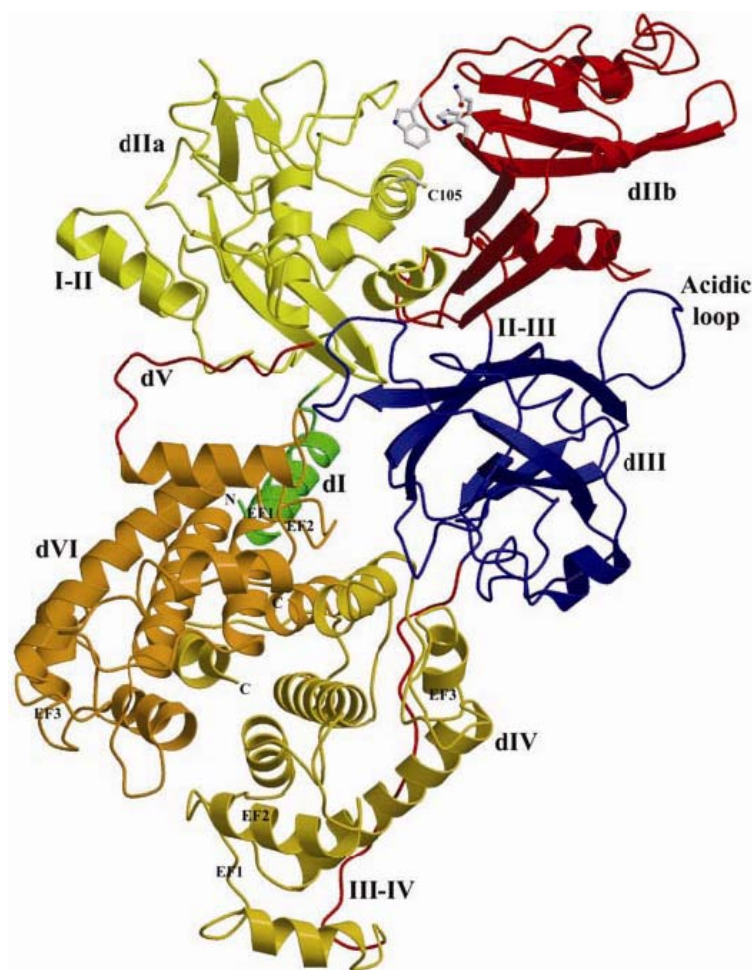


Fig.2 Crystallographic structure of human m-calpain. The 80kDa large subunit starts in the center of the molecule (green,dI). The other domains, in different colors, are labeled dIIa, dIIb, dIII, dIV, dV and dVI. The catalytic residues Cys-105, His-262 and Asn-286 together with Trp-288 are shown with all non-hydrogen atoms(grey) at the top of domain IIb. Figure taken from (Reverter et al., 2001).

1.1.2 Other members of the calpain family

The initial name of the calpain family relates to the calcium dependence of the papain-like, thiol protease activity. The catalytic domain II containing the C-H-N catalytic triad characteristic of cysteine proteases, shares no significant homology with papain or other families of cysteine proteases. Consequently, the calpains have been grouped in a separate class of cysteine peptidases, CLAN CA, family C2(Barrett et al.,

1998), and domain II is thereby the criterion for defining the calpain superfamily. Previous analysis has suggested that the percentage of proteins with similar functions decreases precipitously when sequence identity falls below 35% (Wilson et al., 2000). Accordingly, a homology threshold between domain II candidates of >20-25% sequence identity is recommended to classify a protein as a member of the calpain family (Goll et al., 2003). Additional members of the calpain family have been identified during the past 15 years, including 14 calpain-like genes in mammals, 4 in *Drosophila*, 12 in *Caenorhabditis elegans*, and 14 in *Trypanosoma brucei* (Ersfeld et al., 2005). The expansion of the numbers of calpain-like molecules has led to a degree of confusion as to a unified nomenclature. Several approaches to describe and name calpains and calpain-like molecules have been recommended (Goll et al., 2003, Croall and Ersfeld, 2007). At a first level, calpains are divided into classical and non-classical calpains. The former are characterised by the presence of a four domain structure, including a complete catalytic triad motif in domain II and a penta-EF hand motif in domain IV. All calpains deviating from this pattern are referred to as non-classical calpains. In addition, the following terminology is frequently found in the literature:

Calpain: A general description for proteins comprising the functional unit characteristic of this specific family of cysteine proteases. For example, μ -calpain is a heterodimer comprising a large and a small subunits (as described above); calpain-3 is either a monomer of a 94kDa protein or a homodimer of that protein (Jia et al., 2001).

CAPN or *capn* (*CAPN* in humans and *capn* in other mammals): The gene with sequence relation to the large subunit of the conventional calpains, mostly to the catalytic core domains. For example, *capn1* encodes the catalytic subunits of *calpain-1* (the μ -calpain

in RAT) (Pal et al., 2003).

Cpns (calpain small subunit): Gene encoding a small subunit utilized by some calpains, for example the m-calpain small subunit is called *cpns1*.

CALP (Calpain-Like Protein): Some *capns* lack the key catalytic residues in domain II. They are unlikely to function as cysteine proteases, such as the genes in *T.brucei* (Ersfeld et al., 2005). They are also referred to as pseudo-calpains.

Because this thesis is concerned with capain-like proteins in *T.brucei*, all of which are categorised as non-classical calpains, I provide a brief overview about the calpain-like molecules in other organisms, including mammals, *Drosophila* and *Caenorhabditis elegans*.

1.1.2.1 Calpain-like molecules in mammals

The first calpain homolog was reported in 1989, encoding a gene having 51-54% sequence homology at protein level to the 80kDa subunit of classical calpains (Sorimachi et al., 1989). Subsequently, 14 different genes encoding polypeptides with sequence homology to the calpains have been identified (Sorimachi and Suzuki, 2001). Most of them contain the C-H-N catalytic motif in the domain II with only one exception, CAPN6. The CAPN6 in human has a cysteine to lysine substitution in the catalytic triad and is probably proteolytically inactive (Matena et al., 1998). Later studies have demonstrated that the mRNA of CAPN6 is exclusively expressed during embryogenesis predominantly in developing skeletal and heart muscles (Dear and Boehm, 1999). Additionally, five out of 14 members have been identified to be tissue specific because their mRNAs are expressed principally in muscle cells (Sorimachi et

al., 1989, Sorimachi et al., 1993), in the testis (Dear and Boehm, 1999), in the skin during the first 16 days after birth (Dear et al., 2000) or in the testis and lung (Dear and Boehm, 2001). The precise functions of these calpain-like molecules are not entirely clear, although it has been shown that they are involved in cytoskeletal remodeling, signal transduction and cell differentiation (Ono et al., 1998, Sato and Kawashima, 2001).

One non-classical calpain, known as calpain 3 (also known as p94), has attracted particular attention. It was demonstrated that disruption of this gene was the cause of limb girdle muscular dystrophy type 2A (Richard et al., 1995). Calpain 3 was initially shown to be specifically expressed in skeletal muscle cells (Sorimachi et al., 1989), but further analysis using RT-PCR has revealed its presence in cardiac muscle and liver, containing 46% and ~10% as much calpain 3 mRNA as skeletal muscle, respectively (Poussard et al., 1996). Limb girdle muscular dystrophy patients and mice lacking the calpain 3 gene can still form apparently normal muscles during prenatal development. Hence, calpain 3 is not essential for muscle to form but it is a prerequisite for maintaining healthy muscles (Beckmann and Spencer, 2008).

1.1.2.2 Calpain-like molecules in *Drosophila*

A Ca^{2+} -dependent calpain-like activity was first identified in *Drosophila* in 1988 (Pinter and Friedrich, 1988), and a single polypeptide chain of 94kDa was subsequently purified that required 600 μM Ca^{2+} for half-maximal proteolytic activity (Pinter et al., 1992). It has been demonstrated to be proteolytically active without forming a heterodimer with a small subunit (Pinter et al., 1992). The screening of cDNA libraries

in *Drosophila* has identified three genes having homology to the calpain family (Delaney et al., 1991, Jekely and Friedrich, 1999, Theopold et al., 1995). CALPA, a polypeptides of 821 amino acids and a predicted molecular mass of 94kDa (Theopold et al., 1995); CALPB, of 791 amino acids and a predicted molecular mass of 90kDa (Jekely and Friedrich, 1999); CALPC (also called SOL), of 1579 amino acids and a predicted molecular mass of 175kDa (Delaney et al., 1991). Both CALPA and CALPB have the C-H-N catalytic triad as well as the four domain structure similar to the 80kDa subunit of μ - and m-calpains, including the C-terminal five EF-hand motifs, and are therefore grouped as the classic calpains. A recent study suggests CALPA regulates the activity of the NF-kappaB-related transcription factor IkappaB. Knockdown of CALPA increased the levels of the *Drosophila* I kappaB homologue Cactus during embryonic development (Fontenele et al., 2009). CALPC is different from classical calpains (Delaney et al., 1991). Although a C-terminal region between amino acids 1017 and 1320 represents 36.3% identity to the domain II of human μ -calpain, it lacks the C-H-N motif and the N-terminal half of the protein has no homology with the calpains but some similarity to zinc fingers found in DNA-binding proteins. Furthermore, CALPC does not have a domain IV containing EF-hand motifs, and is therefore grouped as a non-classical calpain. CALPC is involved in the development of sensory neurons. Mutations in this gene result in a rudimentary optic lobe in the brain of the fly, due to the absence of certain classes of neurons (Delaney et al., 1991).

1.1.2.3 Calpain-like molecules in *Caenorhabditis elegans*

14 genes coding for non-classical calpain-like proteins have been identified in *C.elegans*,

all lacking the calmodulin-like domain IV. Very little is known about the actual catalytic activity of these proteins as none of them has been purified and specific substrates have not been identified. One of the calpain-like genes, TRA-3 encodes a protein of 648 amino acid with significant sequence homology to domains II and III of μ - and m-calpain (Barnes and Hodgkin, 1996). The C-terminal domain, named domain T, has no homology with domain IV of μ - and m-calpain. However, the N-terminal 41 amino acids of TRA-3 have 49% similarity with the C-terminal region of m-calpain, which is the region involved in the 28kDa-80kDa subunit interaction. However, a small calpain subunit has not been identified in the *C.elegans* genome. Studies have shown that TRA-3 is involved in a pathway leading to female sexual differentiation in a Ca^{2+} -dependent manner (Sokol and Kuwabara, 2000). Interestingly, two homologues of TRA-3 in humans, CAPN5 and CAPN6, also lack the domain IV, and their tissue-specific expression patterns in testis imply potential involvement in sexual development (Dear et al., 1997, Dear and Boehm, 1999). A recent study has revealed that the catalytic activity of TRA-3 is required for the neuronal regeneration in *C.elegans* (Syntichaki et al., 2002).

1.1.2.4 Calpain-like molecules in *Trypanosoma brucei*

Trypanosoma brucei

The protozoan parasite *Trypanosoma brucei* is the causative agent of bovine nagana and human sleeping sickness, a disease estimated to affect 300,000 – 500,000 persons each year in sub-Saharan Africa, killing approximately 50,000 (www.who.int/mediacentre/factsheets/fs259/en/). Chemotherapy is available for African

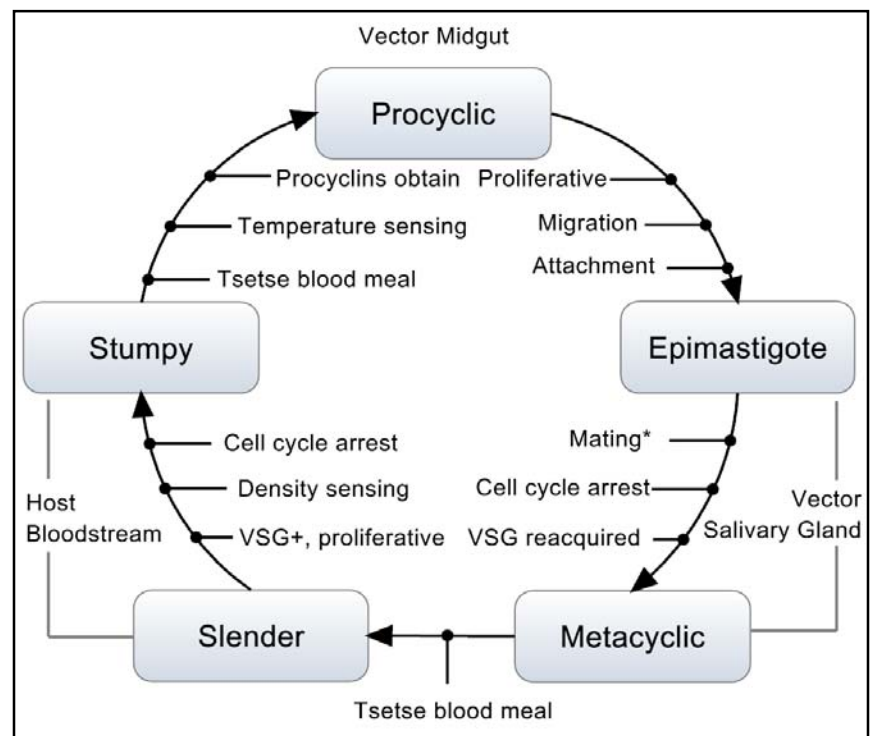
sleeping sickness, but existing drugs are often toxic and can induce adverse effects (Barrett et al., 2003). The high rates of relapse after treatment also indicates the emergence of drug resistance (Legros et al., 1999). New and better drugs are urgently needed.

These parasites have complex life cycles allowing survival within two hosts – an insect vector and a mammalian host (Fig.3). During the blood meal of an infected tsetse fly (*Glossina* spp), the parasites are inoculated into the mammalian host, where they rapidly spread and proliferate (Vickerman, 1985, Vickerman et al., 1988). In the later stage of infection, these quickly dividing cells will invade the central nervous system (CNS) by crossing the blood-brain barrier and are eventually lethal to the hosts if left untreated (Kennedy, 2004). In mammals, the parasite survives extracellularly in the bloodstream by evading antibody responses through antigenic variation, which involves the sequential expression of antigenically different variable surface glycoproteins (VSGs) on the surface of the parasite (McCulloch, 2004, Pays et al., 2004). The proliferative slender forms are induced to differentiate to the cell cycle-arrested stumpy forms through an undefined low-molecular-weight factor (SIF, stumpy induction factor) as parasite numbers increase, suggesting some form of quorum sensing (Matthews et al., 2004). These non-proliferative stumpy forms are crucial for life-cycle progression for two reasons. First, it prolongs host survival by limiting the parasite population density and thereby increases the probability of disease transmission. Second, only the stumpy forms can successfully establish an infection in the insect vector (Matthews, 2005). In the insect, the VSG coat is rapidly lost when the parasite is ingested by the insect and replaced with a procyclin surface protein. Trypanosomes migrate from the midgut to the

fly salivary glands, and attach to the microvilli of epithelial cells via their single flagellum which is structurally modified in the process (Urwyler et al., 2007). Little is known about the migration and how trypanosomes connect to the salivary glands at a molecular level. To complete the trypanosome life cycles, the parasites proliferate in the salivary glands, and eventually give rise to the non-dividing ‘ready-to-infect’ metacyclic forms.

Fig.3 The life cycle of *Trypanosoma brucei*

Proliferative slender forms survive within the host bloodstream, exhibiting antigenic variation as the primary route of immune evasion. Differentiation from the slender forms to non-proliferative stumpy forms limits parasitemia in the host and preadapts the parasite to the life in the tsetse fly. Once in the tsetse midgut, stumpy form parasites reenter the cell cycle and differentiate to the proliferative procyclic form. After



migrating to the salivary gland, parasites attach to the epithelial cells through the flagellum. Eventually, these generate non- proliferative metacyclic forms, which re-acquire a VSG coat and are ready for the transmission to a new mammalian host. A fuller version of the parasite life cycle is detailed in (Vickerman, 1969b).

* Genetic exchange events between trypanosomes have been reported to take place in the tsetse salivary gland. However, the exact process is not fully understood (Bingle et al., 2001, Gibson, 2001)

During the complex life cycles, the parasites undergo a number of biochemical/structural modifications, which are critical for the survival. These differential events require stage-specific changes to basic cell biological processes that are involved in signal transduction, cytoskeleton remodeling, environmental adaptation,

etc (Matthews et al., 2004, Matthews, 2005). Since previous studies in other organisms suggest that the calpains are also frequently involved in these processes, the calpain protein family in *T.brucei* is an attractive candidate, which may contribute to the understanding of differential events.

The calpain-like proteins in T.brucei

The calpain-related gene families in trypanosomatids (*T. brucei*, *T. cruzi*, *Leishmania*) parasites are unusually large. In *T.brucei*, a systematic analysis has revealed 14 calpain-like proteins and 8 small kinetoplastid calpain-related proteins. Only one shows a typical catalytic triad, but none possesses calcium-binding motifs (see below). According to the domain structure and sequence composition, they are categorized into five groups (Fig.4). For a detailed phylogentic analysis and comparison of all calpain members in kinetoplastid, see (Ersfeld et al., 2005)

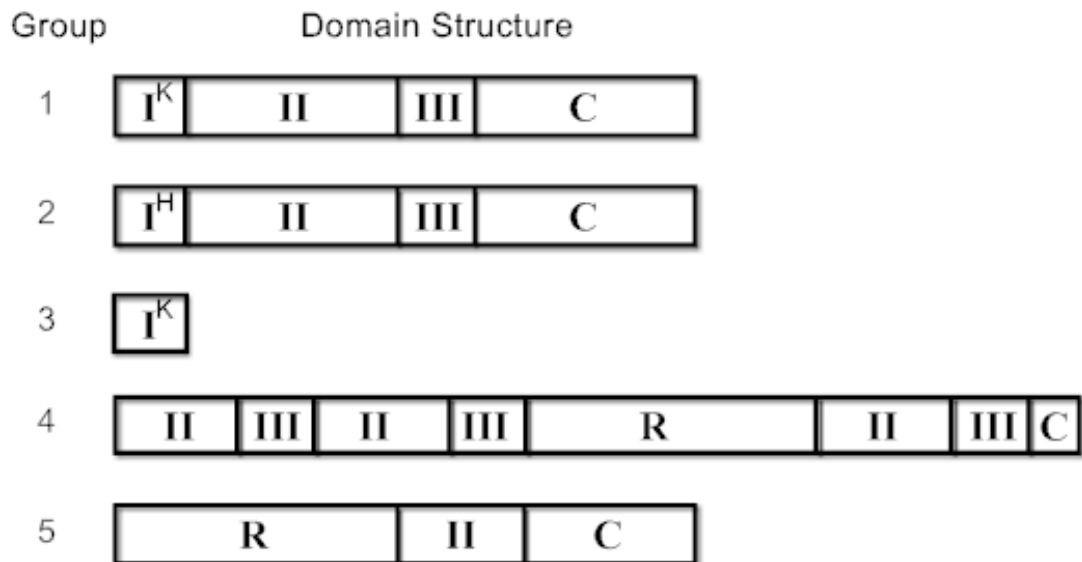


Fig.4 Classification of calpain-like proteins in kinetoplastids according to their domain structures. I^k, kinetoplastids-specific domain I; I^H, heterogeneous domain I; R, repetitive sequence domains; C, C-terminal domain. Figure used with permission (Ersfeld et al., 2005).

Group 1 includes sequences closely related to classic calpains comprising a similar four-domain structure. However, the domain I(I^k) is specifically conserved within kinetoplastids and the variable C-terminal domain IV (termed domain C in kinetoplastids) does not contain any EF-hand motifs.

Group 2 proteins are similar in domain structure to Group 1, except the domain I(I^H) is heterogeneous and unrelated to domain I^k found in Group 1 and 3.

Group 3 is an unusual group and only identifiable as part of the calpain gene family by the shared presence with Group 1 calpain-like proteins of the domain I^K. It only contains the short domain I^k sequence that is exclusively found in kinetoplastids and shows no similarities to any functional motifs. Therefore, the members of group 3 are named as SKCRPs (small kinetoplastids calpain-related proteins). The close relation of group 1 and 3 calpains is also indicated by the observation that genes of group 3 calpains always cluster with genes of group 1, suggesting an origin by multiple rounds of gene duplication events (Ersfeld et al., 2005). The groups 1 and 3 also share the presence of a dual or single N-terminal acylation motif on some of the sequences.

Group 4 proteins contain three repeats of domains II and III. The second and third domain copies are separated by varying numbers of tandem repeats of 70 amino acids unit length, most likely forming a coiled-coil structure.

Group 5 has only one characterized member, consisting of a protein with a number of N-terminal repeats, followed by a domain II and a C-terminal domain.

1.1.3 The multifunctional enzyme family

With the help of many completed genome databases, it is possible to predict and annotate hypothetical genes with potential enzymatic activities through the evolutionary lineage. Some enzyme families are conserved in functionality throughout the evolution (Ellis and Brown, 2009, van Wijk and Timmers). However, detailed studies have revealed that many superfamilies are particularly promiscuous in terms of function, exhibiting not only diversity in enzymatic aspect such as catalytic activity and substrate specificities, but also including homologues which do not function as enzymes at all, so-called pseudoenzymes (Bartlett et al., 2003, Pils and Schultz, 2004b). The definition of “multifunctional enzyme family” is thereby classified by the simultaneous existence of the enzyme and non-enzyme homologues. Elucidating the cellular functions and relationship of the members are important in understanding the entire protein family (Pils and Schultz, 2004b). Notably, it is different from the “moonlighting” proteins: a single multifunctional protein with both catalytic and non-catalytic roles (Jeffery, 1999, Jeffery, 2009). The homologous enzyme and non-enzyme proteins in the Protein Data Bank (Bernstein et al., 1977) have been broadly reviewed (Todd et al., 2001), as well as some multifunctional enzyme families, including tyrosine phosphatase family (Pils and Schultz, 2004a), and cyclin-dependent kinases (CDKs) family (Doonan and Kitsios, 2009). It has been demonstrated that the inactive enzyme-homologues, although devoid of catalytic activity, have adopted new functions that are mainly involved in regulatory processes (Pils and Schultz, 2004a). In the calpain superfamily, the identification of several calpain homologues, especially the calpain-like proteins in kinetoplastids (Ersfeld et al., 2005) clearly suggest it is a multifunctional enzyme family with both

catalytic and non-catalytic members. Therefore, it provides an excellent chance to study the potential regulatory function of the non-catalytic calpain homologues in the well-established model organism *Trypanosoma brucei*, and contribute to the understanding of the entire calpain multifunctional family.

2. Aims

Although a bioinformatics analysis of calpain primary sequence has been published (Ersfeld et al., 2005), the insight of their function roles remains largely unknown. In this study, I have mainly focused on the group 1, 2 and 3 calpain-like proteins. In order to address biochemical and cell biological features, I have analysed the following three main aspects.

- A. mRNA expression patterns of the Groups 1, 2 and 3 calpain-like proteins between cultured bloodstream and procyclic life stages of *T.brucei*.
- B. Definition of the subcellular localisation of several of these proteins using an epitope-tagging approach.
- C. Addressed the potential role of N-terminal acylation in protein trafficking and targeting.

3. Materials and Methods

3.1 Cell culture condition

T.brucei procyclic strain 449, expressing the tet-repressor (Wirtz et al., 1999) was maintained in semi-defined medium SDM79 (Invitrogen) with 15% (v/v) heat inactivated calf serum, 0.3% (w/v in PBS) haemin solution (2.5 mg/ml) and phleomycin (5 µg/ml) at 27°C. The procyclic strain PCF 29-13, expressing the tet-repressor and T7-RNA polymerase (Wirtz and Clayton, 1995) was cultured in the same medium but with hygromycin and G418 at 50 µg/ml and 15 µg/ml, respectively. *T.brucei* bloodstream 427 strain was maintained at 37°C in the HMI9 medium (Invitrogen) supplemented with 10% calf serum (Biebinger et al., 1997).

3.2 Expression constructs and parasite transfection

Open reading frames were amplified from *T. brucei* genomic DNA using appropriate primers in which restriction sites used for subsequent cloning procedures were incorporated (Appendix.1). The digested PCR products were cloned into the pLEW111-*cmymc* (kindly provided by G. Cross) or pHD1700-*cmymc* (kindly provided by C. Colasante and F. Voncken) tetracycline-inducible vectors carrying phleomycin and hygromycin resistant markers, respectively (Wirtz et al., 1999, Colasante et al., 2006). The pHD_GFP vector was modified from pHD1700-*cmymc* by inserting a GFP cassette prior to the *cmymc* epitope tag. All constructs were confirmed by sequencing. Procyclic cells were electroporated (1700V, 3 x 100µS pulse length, 200ms intervals) in ZPFM buffer (132mM NaCl, 8mM KCl, 8mM Na₂HPO₄, 1.5mM MgAc₂, 90µM CaAc₂, pH

7.4) with 10-15µg linearised plasmid DNA (digested with NotI) using the BTX ECM 830 system and standard protocols described previously (Beverley and Clayton, 1993). Transfectants were selected using appropriate antibiotics. Recombinant protein expression was induced with 1µg/ml tetracycline and screened by western blotting using appropriate antibodies. pLEW111-transformed cells were always grown in the presence of 50ng/ml tetracycline because expression of the selection marker and the protein of interest are under the control of a single inducible promoter.

3.3 RNA extraction and qRT-PCR

GeneDB (<http://www.genedb.org/genedb/tryp/>) accession numbers of all genes incorporated in this study are shown in **Table 1**. Kinetoplastid calpain nomenclature is based on the systematic scheme published previously (Ersfeld et al., 2005). Total RNA of procyclic and bloodstream form cells was extracted from $\sim 10^8$ mid-log phase cells using RNeasy miniprep kit (Qiagen) according to the manufacturer's instructions. Total RNA of homogenous slender and stumpy form cells was kindly provided by Prof Keith Matthews. RNA samples were subsequently treated with RNase-free DNase to remove any contaminating genomic DNA. First strand cDNA was synthesized from 1µg of total RNA using the Omniscript Reverse Transcription kit (Qiagen) and oligo(dt) primers (eurofins MWG). The cDNA reaction was diluted to a working concentration of approximately 20 ng/µl, and used as the template for individual PCR reactions. For qRT-PCR, 2µl of cDNA template was used in a 20µl reaction containing 8µl of H₂O, 10µl 2x SYBR Green mix (ABgene), and 0.4µM gene-specific forward and reverse primers (Appendix 2). qRT-PCR reactions were performed in an iCycler iQtm detection

system (BIO-RAD) with the following cycling conditions: 95°C 15mins initial template denaturation, 40 cycles of 95°C 40sec, 50-55°C 40sec (see Appendix 2 for precise annealing temperatures), 72°C 1min, and a final melting curve from 55-95°C to confirm the presence of a specific PCR product (see Appendix 3 for an example). Gene-specific primers were designed and validated using PrimerPremier 5.0 (Singh et al., 1998) to amplify a ~110bp fragment at the 3' end of the open reading frame, sometimes extending into the 3' untranslated region. The $\Delta\Delta\text{CT}$ method was used to evaluate the data (Livak and Schmittgen, 2001). A PCR fragment amplified from cDNA encoding the paraflagellar rod protein A (PFRA) was used as normalization control (Brenndorfer and Boshart, 2010).

3.4 Protein immunoblotting

All samples for SDS gel electrophoresis were prepared by addition of hot sample buffer (62.5 mM Tris-Cl, pH 6.8, 10% (v/v) glycerol, 2% (w/v) SDS, 0.36 M β -mercaptoethanol) to cell pellets or precipitated protein and heated for 5 min at 95°C. The equivalent of $\sim 10^6$ cells/lane was loaded on sodium dodecyl sulfate-polyacrylamide gels for protein analysis. Electrophoresis and western blotting were performed using standard protocols. Proteins were detected with anti-*cmyc* mAb (clone 9E10, ECACC) antibody or appropriate polyclonal antibody. Chemiluminescence was used for blot development (Western Lightning, Perkin Elmer Life Sciences).

3.5 Hypotonic/Sonication lysis & detergent extraction analysis

Cell fractionation was performed at 4°C in the presence of mammalian protease

inhibitor cocktail (Sigma). For detergent extraction, 2×10^7 cells were washed with 1ml phosphate-buffered saline (PBS) once, centrifuged at 3000xg for 3 min and pellets resuspended in 150 μ l cold lysis buffer (1% (v/v) Triton X-100, 25mM HEPES, 1mMEDTA, pH7.4). After 30 min incubation on ice, lysate was centrifuged at 14,000 x g for 5 min. The detergent insoluble pellet (D_p) was resuspended directly into 20 μ l hot sample buffer. The detergent soluble phase (D_s) was precipitated with 9 volumes of ice-cold acetone overnight and centrifuged at 14,000 x g for 10 min. To the pellet, 20 μ l hot sample buffer was added. For hypotonic/sonication extraction, 2×10^7 cells were washed with PBS and incubated on ice in hypotonic lysis buffer (10mM Tris-Cl, pH 7.4) for 10 min. Lysates were sonicated for 2 x 5 sec with 1min cooling on ice between bursts (Sonicator type 7533A, Dawe Instruments Limited, output 50W). Samples were centrifuged at 14,000 x g for 30 min at 4°C and supernatants were precipitated with acetone. The sonication insoluble pellet (S_p) and sonication soluble fraction (S_s) were prepared by the addition of 20 μ l hot sample buffer. All the samples were heated for 5 min at 95°C and immunoblotted as described above.

3.6 Immunofluorescence microscopy

For immunofluorescence microscopy, $\sim 10^7$ cells were washed once with PBS and resuspended in 500 μ l PBS. An equal volume of 7.2% (w/v) formaldehyde in PBS was added in order to obtain a final concentration of 3.6% (w/v). After incubation on ice for 30 min, cells were then settled onto poly-lysine-coated slides and permeabilised with 0.1% (v/v) Nonidet P-40 in PBS for 10 min, and then treated with 0.1M glycine in PBS for 10 min. Cells were incubated with anti-cMyc monoclonal antibody for 1 h followed

by three washes in PBS and subsequently developed with a FITC-conjugated anti-mouse secondary antibody (Sigma). Cells were embedded in Vectashield (Vector Laboratories) containing 100ng/ml of 4-, 6-diamino-2-phenylindole (DAPI). Images were viewed on a Nikon Eclipse 80i epifluorescence microscope, equipped with a CoolSNAPcf CCD camera (Photometrics) using MetaVue imaging software (Molecular Dynamics) and pseudocoloured in Adobe Photoshop CS3.

3.7 Acyl-biotin exchange assay

The protocol of detecting protein palmitoylation using acyl-biotin exchange assay was first used in yeast (Wan et al., 2007) and adapted to *T.brucei* recently (Emmer et al., 2009). The schematic of the acyl-biotin exchange assay is showed in Fig.5. A Briefly, 1×10^9 mid-log phase parasites were harvested, washed twice with PBS, and then resuspended in 6ml of N-Ethylmaleimide (NEM) lysis buffer (1.7% Triton X-100, 50mM Tris-Cl, 150mM NaCl, 5mM EDTA, 10mM NEM, pH7.4) supplemented with protease inhibitor cocktail (Sigma). Suspensions were incubated for 1 hr at 4 °C with end-over-end rotation for sufficient lysing. Intact cells and nuclei were pelleted at 600 x g for 5mins. Six ml supernatant was split into 3ml aliquots and chloroform-methanol (CM) precipitated (Wessel and Flugge, 1984). The protein pellets were air dried for 3-5mins and resuspended in 600µl of solubilization buffer (50mM Tris-Cl, 5mM EDTA, 4%SDS, pH7.4) supplemented with 10mM NEM. Samples were incubated at 37°C for 10 min with occasional vortexing to facilitate protein resuspension. Suspensions were then diluted with 2.4ml of 0.2% Triton-lysis buffer (0.2% Triton X-100, 50mM Tris-Cl, 150mM NaCl, 5mM EDTA, pH7.4, protease inhibitor cocktail), and incubated

overnight at 4°C with rotation. The next day, NEM was removed by three sequential CM precipitations. For the first and second precipitation, samples were resuspended in 750µl of solubilization buffer (without NEM) and diluted with 2.25ml of 0.2% Triton-lysis buffer. For the third precipitation, pellets were resuspended in 600µl of solubilization buffer, mixed and equally split for experimental and control samples. To one 600µl control sample 2.4ml Tris-label reagent was added (50mM Tris-HCl, 1mM HPDP-Biotin (N-[6-(Biotinamido)hexyl]-3'-(2'-pyridyldithio)-propionamide), 5mM EDTA, 0.2% Triton X-100, pH7.4, protease inhibitor cocktail). To the other sample 2.4ml hydroxylamine-label reagent was added (700mM hydroxylamine, 1mM HPDP-Biotin, 5mM EDTA, 0.2% Triton X-100, pH7.4, protease inhibitor cocktail). Samples were incubated at room temperature for 1 hr with end-over-end rotation. The labeling reagent was removed by three consecutive CM precipitations. The first two precipitations were performed same as for NEM removal. Following the final precipitation, pellets were resuspended in 150µl of 2% SDS buffer (50mM Tris-Cl, 5mM EDTA, 2%SDS, pH7.4) and 30µl was collected as the input (I) sample. The remaining 120µl sample were diluted with 2.28ml of lysis buffer (50mM Tris-Cl, 150mM NaCl, 5mM EDTA, pH7.4). 40µl of streptavidin-agarose beads suspension, pre-washed with pulldown wash buffer (lysis buffer supplemented with 0.2%Triton X-100 and 0.1% SDS), was added to this sample and incubated at room temperature for 90 min with rotation. Beads were collected by centrifugation and washed five times with pulldown wash buffer. Eluate(E) samples were prepared by incubating beads with 22.5µl pulldown elution buffer (pulldown wash buffer with 1% β-mercaptoethanol). Both input and eluate samples were acetone precipitated as described in 3.5, and resuspended in appropriate volume of

1 x SDS-sample buffer for immunoblotting analysis.

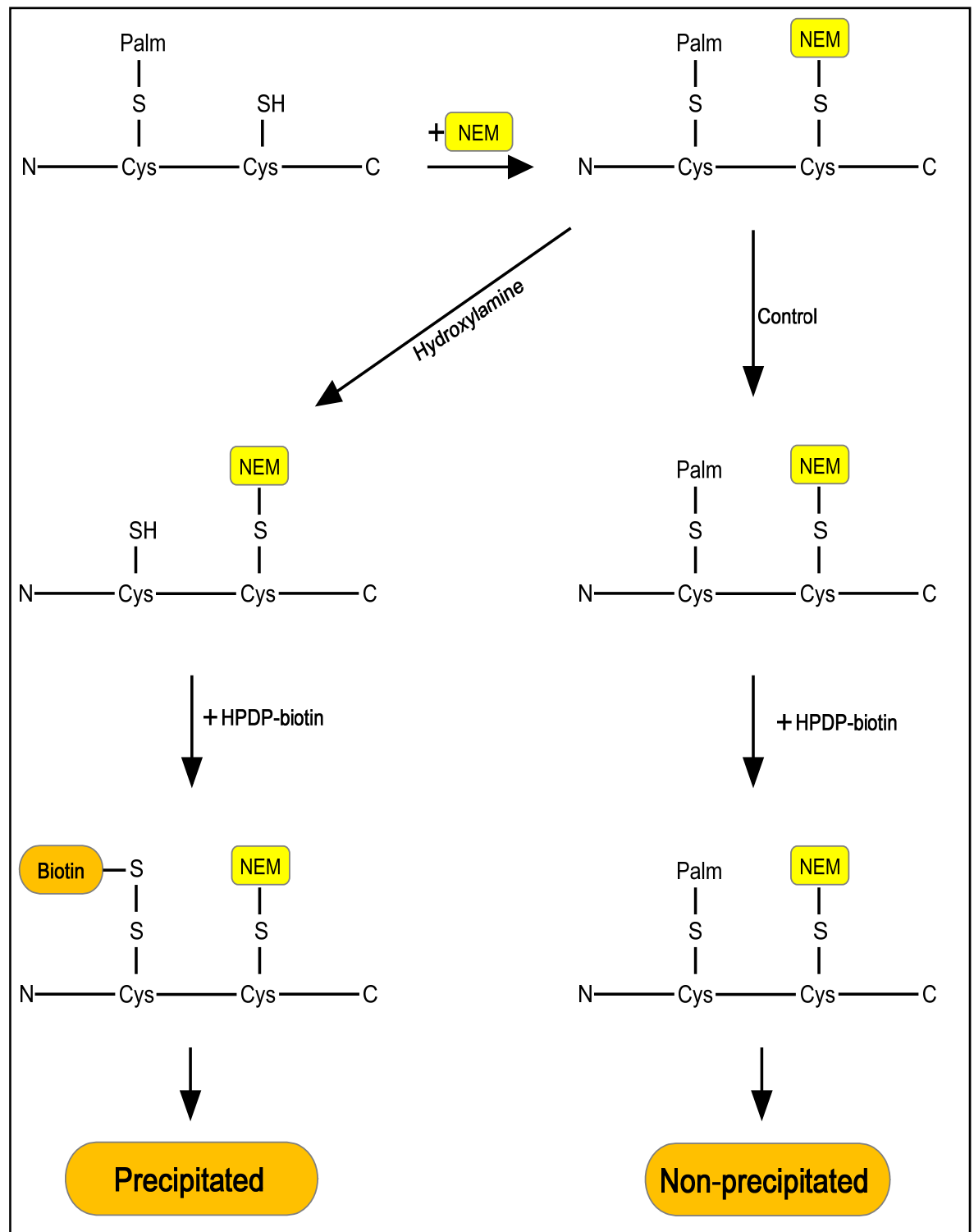


Fig.5 Schematic of the acyl-biotinyl exchange methodology

4. Analysis mRNA expression levels of calpain-like proteins in procyclic and bloodstream trypanosomes.

4.1 Introduction

4.1.1 Control of gene expression in *T.brucei*

Kinetoplastid parasites, such as *Trypanosoma brucei*, represent an early diverging branch of the eukaryotic lineage, undergoing complex life cycles in the mammalian host and the Tsetse fly vector (Reviewed in (Matthews, 2005). Morphological and biochemical adaptations critical for life-cycle progression are reflected in the differential expression of many genes. However, the organization of trypanosome chromosome is extremely unusual. The open reading frames are clustered into long polycistronic transcriptional units. In each cluster, genes that are next to each other share a common transcriptional orientation and are co-transcribed by RNA polymerase II (Clayton, 2002). Individual mRNAs are cleaved from the precursor by the trans splicing reaction, adding a spliced leader (SL) to the 5'-end, and polyadenylation at the 3'-end (Liang et al., 2003). Hence, the promoter-driven control of transcription is probably precluded due to the special gene organization (Teixeira and daRocha, 2003).

The analysis of cohorts of mRNAs involved in functionally co-ordinated processes is now providing a useful tool for the identification of regulatory signals controlling gene expression in eukaryotes (Moore et al., 2005). Very few potential regulatory transcription factors have been identified in the *T.brucei* genome (Palenchar and Bellofatto, 2006) and so far there is no evidence for differential regulation of RNA polymerase II transcription of individual genes or gene clusters (Clayton, 2002).

Therefore, post-transcriptional control is the major factor regulating differential gene expression (Clayton, 2002). However, two exceptions have been reported. Variant surface glycoproteins (VSGs) of bloodstream forms and EP/GREET procyclins of the insects forms are the major surface proteins of *T.brucei*, and their precise regulation is critical for life cycle progression. During *in vitro* synchronous transition from bloodstream stumpy forms to procyclic forms, the VSG coat is lost after 4-5 hours and the insect-specific EP procyclin coat is gained after 2 hours (Matthews, 1999). In contrast to most protein coding genes, which are transcribed by RNA polymerase II, both VSG and procyclin genes are transcribed by RNA polymerase I (Gunzl et al., 2003). One explanation is probably the high transcription rate: a chromosomally integrated reporter gene transcribed by polymerase I yields 10 times as much product as the identical gene transcribed by polymerase II (Biebinger et al., 1996). Since the major surface proteins are particularly abundant, RNA polymerase I with higher transcriptional efficiency may be preferable.

Most genes in *T.brucei* are regulated post-transcriptionally, mainly at the level of mRNA stability (Clayton, 2002). In several cases the regulation of mRNA stability has been examined for some stage-specific genes. The VSG mRNAs 3'-UTR contain 8mer and 14mer nucleotide elements which can stimulate bloodstream-form specific expression and suppress insect-form expression (Berberof et al., 1995). More detailed characterization has been performed for the insect form surface protein genes, encoding EP/GREET procyclins. Two elements have been characterized in their 3'-UTRs: a stem-loop 16mer contributing to mRNA stability and translation efficiency (Furger et al., 1997) and a single-stranded U-rich 26mer mediating developmental regulation (Hotz et

al., 1997, Schurch et al., 1997). However, being transcribed by RNA polymerase I make VSG and procyclin genes different from the majority of regulated trypanosome genes. The phosphoglycerate kinase B (PGKB) is a procyclic-specific gene transcribed by RNA polymerase II. Studies in its 3'-UTR region revealed a similar U-rich 26mer element, deletion of which abolished the stage-specific regulation at both RNA and protein levels (Blattner and Clayton, 1995, Clayton, 2002). These sequences bear a strong resemblance to the destabilizing AU-rich elements (AREs) found in the 3'UTR of many mammalian mRNAs involved in cell growth and differentiation (Mitchell and Tollervey, 2000). A systematic analysis has further identified a conserved [TATTTTTT] signal within the 26mer-related elements when analysing the 3'-UTRs of mRNAs coding for proteins of the mitochondrial cytochrome oxidase (COX) complex, a protein complex that is specifically upregulated in procyclic cells. An alignment with additional 179 procyclic up-regulated genes identified that 30% of their transcripts contained the [TATTTTTT] elements (Mayho et al., 2006).

4.2 Results

4.2.1 Calpain gene expression in bloodstream and procyclic forms

In order to have an overview of the differential expressions of calpain-like proteins in *T.brucei*, we have compared the mRNA expression levels of Group 1, 2 and 3 calpains in procyclic and bloodstream forms. Nineteen calpain-like proteins have been analyzed (**Table 1**). The selection was based on a detailed phylogenetic analysis of calpain-like proteins that has been presented previously (Ersfeld et al., 2005). The mRNA level of CALP4.1/CAP5.5 was 6.1 times upregulated in procyclic forms, consistent with previous Western and Northern blot analysis (Hertz-Fowler et al., 2001). By using

CALP4.1/CAP5.5 as a standard for a differentially expressed gene, and the PFRA, encoding for a structural protein of the paraflagellar rod, as a non-differentially expressed gene, genes with differential mRNA ratios equal to or above 4 were deemed to be differentially expressed, with stage-specific expression of the corresponding proteins highly likely. Using this threshold, five genes out of the nineteen investigated exhibited differential expression patterns.

A BSF/PCF mRNA ratio of approx 15 indicated that Group 1 CALP8.1 is expressed only in BSF. This corresponds well with a BSF/PCF ratio of 19 for CALP8.1 reported by Olego-Fernandez et al., also determined by quantitative RT-PCR (Olego-Fernandez et al., 2009). This protein is very similar in sequence to CALP4.1/CAP5.5. A detailed description of this bloodstream-specific protein, also named CAP5.5V (for variant) by these authors to emphasize its close relation to CAP5.5, has recently been published (Olego-Fernandez et al., 2009). Three out of eight SKCRPs (Group 3) were differentially expressed: SCKRP5.1 had the BSF/PCF mRNA ratio of 4.4 indicating higher expression levels in bloodstream forms. SKCRP1.6 and SKCRP7.2 were upregulated in procyclic with the PCF/BSF mRNA ratios of 4.5 and 9.8, respectively.

4.2.2 TbCALP1.3 equally expressed in procyclic, slender and stumpy forms

To test the gene expression in slender and stumpy forms, the RNA isolated from homogenous slender and stumpy form cells were used as the template. TbCALP1.3 was subjected to a further analysis comparing its mRNA levels of three distinct life stages using the Pfaffl method (Pfaffl, 2001). The ratios are calculated by the following formula:

$$Ratio = \frac{(E_{CALP1.3})^{\Delta Ct_{-CALP1.3}}}{(E_{PFR-A})^{\Delta Ct_{-PFR-A}}}$$

where E is the primers' amplification efficiency, which is assigned to a numerical value of 2 in the $\Delta\Delta CT$ method (the efficiency is set to 100% in $\Delta\Delta CT$ method).

The primer efficiency of CALP1.3 and PFR-A has been determined by the standard curves (Appendix 4), with the value of 84.9% and 99.8%, respectively. The outcome shows equal mRNA level ratios of 1.23 : 1.39 : 1.17, corresponding to procyclic, slender and stump forms, suggesting CALP1.3 is probably expressed at the same level throughout the lifecycles.

Name	GeneDB ID	Group	Domains	QPCR ratios (Mean \pm SD)	
				PF	BSF
TbCALP 1.1	Tb927.1.2100	1	I ^k , II, III, C	1.3 \pm 0.03	1
TbCALP 1.2	Tb927.1.2110	1	I ^k , II, III, C	1.7 \pm 0.23	1
TbCALP 1.3	Tb927.1.2120	1	I ^k , II, III, C	1.4 \pm 0.20	1
TbCALP 4.1	Tb927.4.3950	1	I ^k , II, III, C	6.0* \pm 3.01	1
TbCALP 4.2	Tb927.4.3940	1	I ^k , II, III, C	1	2.0 \pm 0.18
TbCALP 8.1	Tb927.8.8330	1	I ^k , II, III, C	1	15* \pm 3.69
TbCALP 9.1	Tb09.160.5550	1	I ^k , II, III, C	1.4 \pm 0.84	1
TbCALP 6.1	Tb927.6.3310	2	I ^H , II, III, C	1	1.7 \pm 0.26
TbCALP 10.1	Tb10.389.0470	2	I ^H , II, III, C	1	2.8 \pm 1.05
TbCALP 10.2	Tb10.70.5950	2	I ^H , II, III, C	1	2.4 \pm 0.36
TbCALP 11.3	Tb11.57.0008	2	I ^H , II, III, C	1	2 \pm 0.07
TbSKCRP 1.4	Tb927.1.2150	3	I ^k	1.6 \pm 0.18	1
TbSKCRP 1.5	Tb927.1.2160	3	I ^k	1	1.3 \pm 0.14
TbSKCRP 1.6	Tb927.1.2230	3	I ^k	4.5* \pm 1.07	1
TbSKCRP 1.7	Tb927.1.2260	3	I ^k	1	1.8 \pm 0.38
TbSKCRP 5.1	Tb927.5.2190	3	I ^k	1	4.4* \pm 0.76
TbSKCRP 7.1	Tb927.7.4060	3	I ^k	2.2 \pm 0.30	1
TbSKCRP 7.2	Tb927.7.4070	3	I ^k	9.8* \pm 2.61	1
TbCALP 10.3	Tb10.70.5060	3	I ^k	1	2.3 \pm 0.15

Group 1:

I ^k	II	III	C
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Group 2:

I ^H	II	III	C
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Group 3:

I ^k

Table.1: Differential mRNA expression levels of calpain-like genes between the procyclic and bloodstream forms of *T.brucei*. Transcripts were validated by qRT-PCR and the resulting ratios between PCF and BSF are given. Transcripts with a more than 4-fold difference are indicated by an asterisk. Systematic names, GeneDB accession numbers, calpain group and domain structure are given. The lower panel is a representation of the domain structure of calpain-like proteins investigated in this study. I^k, kinetoplastid-specific domain I; I^H, heterogeneous domain I; C, C-terminal domain IV unrelated to domain IV of typical calpains (Ersfeld et al., 2005) for details)

4.3 Discussion

We have compared the transcriptional status of 11 calpain-like proteins (CALPs) and 8 small kinetoplastid calpain related proteins (SKCRPs) in cultured procyclic and bloodstream forms of *T.brucei*. Two transcripts, SKCRP5.1 and CALP8.1, were differentially expressed in bloodstream cells. Three transcripts, SKCRP1.6, SKCRP7.2 and, as was described before and used here as a quality standard, CALP4.1/CAP5.5, were differentially expressed in procyclic forms.

SKCRP5.1 contains no known functional domains but it shares the *META-1* gene homologous domain with some other kinetoplastid proteins (Uliana et al., 1999, Ramos et al., 2004). In *Leishmania*, two proteins containing the META-1 domain, one with (META-2) and one without (META-1) the calpain domain I^K, are upregulated in the infective metacyclic promastigotes insect stage (Ramos et al., 2004). In *T.brucei*, the *META-1* homologue is upregulated in procyclic cells and the gene is located in close proximity to the *SKCRP5.1* gene, separated only by one unrelated ORF (Jones et al., 2006).

T.brucei CALP4.1/CAP5.5 and CALP8.1/CAP5.5V are closely related based on sequence similarity and biochemical features: protein sequences are 74% identical, the N-terminal sequence around the dual acylation motifs is identical and domains I, II, III are highly conserved (Olego-Fernandez et al., 2009). However, they are expressed in different life cycle stages. A comparable situation has been described for the closely related *T. brucei* microtubule-associated proteins CAP15 and CAP17 (Vedrenne et al., 2002). CAP15 is predominantly, but not exclusively, expressed in bloodstream forms and CAP17 is found only in procyclic forms.

It is not clear whether CAP5.5/CALP4.1 and CALP8.1/CAP5.5V are functionally equivalent or whether sequence differences confer life cycle-specific functions (Matthews, 2005). Recently published data described severe perturbations of cytokinesis in both life-cycle stages after RNAi-mediated depletion of both proteins, nuclear mispositioning, irregular flagellar pocket biogenesis and disordered appearance of the subpellicular microtubule corset (Olego-Fernandez et al., 2009), suggesting their functions may be comparable.

Pairs of highly conserved genes with divergent stage-specific expression patterns are also present in the group of SKCRPs. SKCRPs1.6 & 1.7 and SKCRPs 7.1&7.2 protein sequences are very similar (Appendix 5). The flanking 5' and 3' UTR regions are, however, different between all genes. In these pairings only one in a pair exhibits life cycle stage specificities: SKCRP1.6 and 7.2 are both upregulated in procyclic forms, whereas SKCRPs 1.7 and 7.1 show no differential transcript levels.

A U-rich core containing TATTTTTT sequence is present in about 30% procyclic up-regulated proteins (Mayho et al., 2006). CAP5.5, SKCRP1.6 and SKCRP7.2 contain a U-rich region in the 3'UTR, however, none of them comprises the "TATTTTTT" element or the 26mer U-rich sequence, suggesting alternative regulation processes may account for the procyclic specificity.

A recent microarray study has been performed to establish the transcript differential expressions between different life cycle stages in *T.brucei* (Veitch et al., 2010). Two stage-specific transcripts were included. SKCRP1.6 was 4.2 times upregulated in procyclic forms, and CALP8.1 was 21 times upregulated in bloodstream cells, consistent with our data. However, CALP4.1/CAP5.5 was shown to be evenly expressed

in the microarray study, and two stage-specific transcripts, SKCRP5.1 and 7.2, were not included. We have used a critical threshold (4 times difference) to minimize possible artifacts and experimental variations when determining stage specificity. This is because previous studies have shown that the CALP4.1/CAP5.5 is procyclic-specific at the protein level (Hertz-Fowler et al., 2001), and represented a 4 times difference at the mRNA level in this study. However, the mRNA levels do not always correspond to the protein presence in *T.brucei*. The bloodstream form has a rudimentary mitochondrion and many transcripts encoding mitochondrial proteins are down-regulated (Brems et al., 2005, Koumandou et al., 2008). However, although the cytochrome c mRNA level is stable and equally present in both life stages, the encoded protein cannot be detected in bloodstream forms (Torri et al., 1993). Therefore, our data from mRNA analysis do not necessarily reflect the differential expressions at the protein level and further experiments at protein level (e.g. using antibodies) must be performed to confirm the stage specificity.

5. Localization and membrane association of calpain-like proteins

5.1 Introduction:

Classical calpains, including both ubiquitous and tissue-specific isoforms displayed diverse subcellular localizations (Goll et al., 2003). The association with several subcellular organelles has been confirmed, including endoplasmic reticulum (ER), Golgi apparatus and mitochondria (Gopalakrishna and Barsky, 1986, Hood et al., 2003, Garcia et al., 2005). Subsequently, various sub-mitochondrial localizations have been identified. A mitochondrial calpain has been identified in both the intermembrane space (IMS) and the matrix of swine liver mitochondria (Ozaki et al., 2007). The μ -calpain is localized to the mitochondrial IMS of rat neuroblastoma cells, modulated by its N terminus signal (Badugu et al., 2008). Furthermore, calpains can also exhibit a dynamic localization in a Ca^{2+} -dependent manner (Hood et al., 2004). For instance, calpain B is mainly in the cytoplasm at low Ca^{2+} concentration in *Drosophila*, but adheres to intracellular membranes when Ca^{2+} increases (Farkas et al., 2004).

In kinetoplastids, the localization and organelle association of the calpain-like proteins remains largely uncharacterized. Very few calpain proteins have been experimentally characterized. CALP4.1/CAP5.5 (Group 1 calpain-like protein in *T.brucei*) was found to be associated with the subpellicular cytoskeleton (Hertz-Fowler et al., 2001). Depletion of CALP4.1/CAP5.5 by RNA interference results in disorganization of the sub-pellicular microtubules and organelle mis-positioning (Olego-Fernandez et al., 2009). Biochemically, its N-terminal region contains a dual acylation motif: glycine 2

being myristoylated followed by cysteine 3 being palmitoylated, which usually mediates protein-membrane associations (Resh, 1999, Ersfeld et al., 2005). *In vivo* labelling of CALP4.1/CAP5.5 (Group 1) and the calpain-related protein SMP-1 (Group 3) in *Leishmania major* with radioactive fatty acids has confirmed the presence of these fatty acid modifications (Hertz-Fowler et al., 2001, Tull et al., 2004). N-terminal acylation motifs of calpain-like proteins are unique in the kinetoplastids and are only present in some Group 1 and 3 members harbouring the kinetoplastids-specific domain I^k. Apart from CALP4.1/CAP5.5, there are six calpain-like members with predicted N-terminal acylation motifs in *T.brucei*. In this chapter, I have examined the subcellular localization of ten CALPs/SKCRPs, including those with predicted N-terminal acylations, and analyzed the protein-membrane associations using a hypotonic/detergent extraction method.

5.2 Results:

5.2.1 Membrane association of calpains

Using bioinformatics tools we have shown previously the presence of potential N-terminal acylation sites of some calpain-like proteins from Groups 1 and 3 (Ersfeld et al., 2005). Protein acylation is important to facilitate the association with cellular membranes (Magee and Seabra, 2005). In order to investigate possible protein-membrane association of potentially acylated *T.brucei* calpain-like proteins, we distinguished between membrane-associated and non-associated proteins by using hypotonic cell lysis in the absence of detergents, which leaves membrane-associated proteins insoluble in the pellet fraction. However, if cells are lysed with the non-ionic

detergent Triton X-100, membrane-associated proteins are solubilised. Throughout this study, we used C-terminally c-myc tagged, ectopically expressed genes cloned into an inducible expression system, with the exception of a monoclonal antibody that was available against CALP4.1/CAP5.5, serving as a control (Hertz-Fowler et al., 2001). A shift from insolubility after hypotonic lysis without detergent to solubility in the presence of detergent is consistent with an association to cellular membranes. In this study, ten calpain-like proteins were analysed including six with predicted acylation sites (Fig. 6).

CALP1.3^{myc}, SKCRP1.5^{myc}, SKCRP1.6^{myc} and SKPCR1.7^{myc} all have dual acylation motifs and are hypotonic/sonication lysis-resistant but detergent-soluble, indicating membrane association. SKCRP1.4^{myc} has only a single acylation motif for myristoylation, but fractionates like a membrane-associated protein. CALP4.2^{myc} and CALP6.1^{myc} also remained insoluble after hypotonic/sonication lysis and solubilised in the presence of Triton X-100, although there was no predicted N-terminal acylation site. Two proteins without acylation motifs, SKCRP7.1^{myc} and SKCRP7.2^{myc}, share almost identical sequences. After hypotonic/sonication lysis, the bulk of both proteins were in the soluble fraction, indicating that these two proteins are not membrane-associated. However, a small proportion of both proteins was recovered in the pellet fraction after hypotonic/sonication lysis, but not after detergent extraction. This could indicate, in conjunction with our observation that some immunostaining of SKCRP7.2^{myc} is observed at the flagellar tip (see below), that SKCRP7.2 is associated with the flagellar membrane. For the same reason, an association of the wild-type SKCRP7.1 with membranes of the cell body cannot be excluded.

The procyclic-specific protein CALP4.1/CAP5.5 has been experimentally demonstrated to be cytoskeleton-associated and to be dually myristoylated and palmitoylated (Hertz-Fowler et al., 2001). This association to the cytoskeleton renders CALP4.1/CAP5.5 Triton X-100-insoluble and resistant to hypotonic lysis. CALP8.1^{myc}, the bloodstream-specific calpain-like protein with a sequence very similar to CALP4.1/CAP5.5, showed extraction characteristics indistinguishable to those of CALP4.1/CAP5.5.

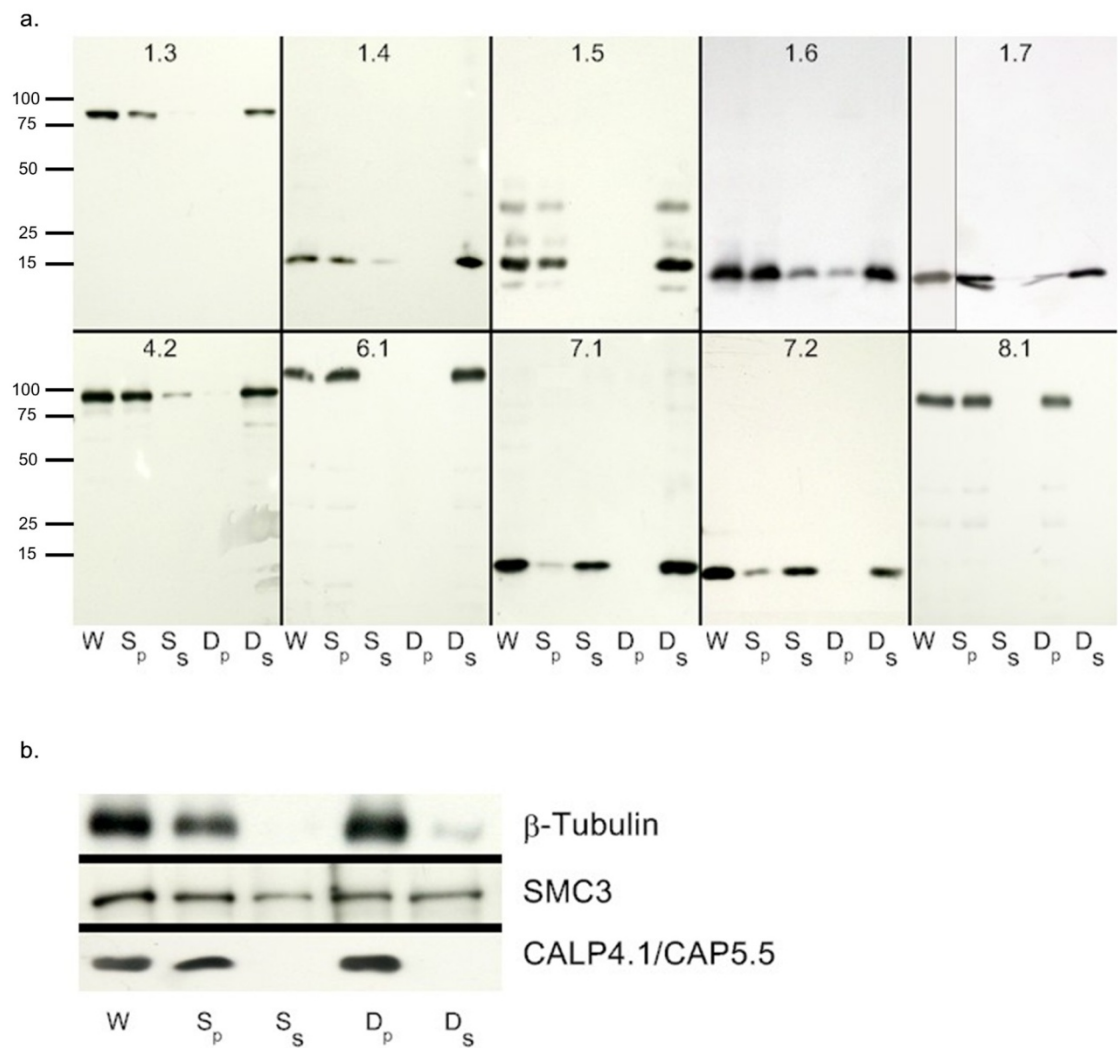


Fig.6

a. Cell fractionation analysis of calpain-like proteins. Whole cell extracts(W), sonication-insoluble fraction(S_p), sonication-soluble fraction(S_s), detergent-insoluble(D_p) and detergent-soluble fraction(D_s). The whole cell extract of SKCRP1.7 was loaded on a separate membrane. Image was taken and transformed into Fig.5a

b. β -tubulin, SMC3 and CALP4.1/CAP5.5 are used as controls for validating the efficiency of extraction. The cohesin subunit SMC3 of *T. brucei* has been shown to consist of a chromatin-associated fraction (insoluble by hypotonic/sonication lysis or detergent-extraction) and a non-chromatin-associated, soluble fraction (Bessat and Ersfeld, 2009).

5.2.2 Cellular localisation of calpain-like proteins

To identify the cellular locations of calpain like proteins in *T.brucei*, the same cmc-tagged expression constructs used for the biochemical analysis were employed in order to localize the tagged proteins by immunofluorescence microscopy. Expression of fusion proteins of predicted size was confirmed by Western blotting using anti-cmc antibodies (Fig.6a). Because overexpression of fusion proteins can potentially cause mistargeting, time courses of protein expression were taken and data recorded when the proteins were first readily detectable by blotting or microscopy, usually 1-2 hours post-induction (Fig.8a). Time courses of expression of CALP1.3^{cmc} and SKCRP1.5^{cmc} are shown in Fig.7, demonstrating that only at later time points after induction CALP1.3^{cmc} accumulates inside the entire flagellum and SKCRP1.5^{cmc}, in addition to its flagellar localization, accumulates in the cell body.

CALP1.3^{cmc} was localised at the tip of the flagellum, whereas SKCRP1.5^{cmc} was located along the entire flagellum (Fig.8a). Because the exclusive localisation of TbCALP1.3 at the flagellar tip has potentially important functional implications, a mouse polyclonal antibody was generated against the recombinant wild-type CALP1.3 protein and showed that the wild-type protein localised, exactly as the cmc-version, at the tip of the flagellum (Fig.9).

SKCRP1.4^{cmc}, which has similar *in vitro* fractionation characteristics to CALP1.3^{cmc} and SKCRP1.5^{cmc}, was homogeneously distributed throughout the cell body (Fig.8a). Also, both SKCRP7.1^{cmc} and SKCRP7.2^{cmc} produced a staining of the entire cell body. However, SKCRP7.2^{cmc} displayed an additional signal at the tip of flagellum (arrow in Fig.8a), whereas SKCRP7.1^{cmc} was completely excluded from the flagellum. The

membrane fraction-associated proteins CALP4.2^{myc} and CALP6.1^{myc} both produced a homogeneous staining of the whole cell body and were excluded from the flagellum. The absence of a more intense staining around the cell periphery points to the potential association with components of the endomembrane system.

CALP8.1^{myc} was ectopically expressed in bloodstream forms as it was shown to be a BSF-specific transcript. Like the previously characterised cytoskeleton-associated protein CALP4.1/CAP5.5, CALP8.1^{myc} was also associated with the subpellicular cytoskeleton as judged by its enrichment around the outer perimeter of the cell body and its resistance to *in situ* extraction of cells with non-ionic detergent (Fig. 8b). Therefore, our data provide direct biochemical and cell biological evidence that CALP8.1/CAP5.5V is indeed, as hypothesized by Olego-Fernandez et al. based on sequence comparison with CALP4.1/CAP5.5 and phenotypic observation after RNAi-mediated depletion (Olego-Fernandez et al., 2009), a cytoskeleton-associated protein with characteristics indistinguishable from CALP4.1/CAP5.5. Furthermore, when expressed in procyclic cells, localisation and detergent-resistance of CALP8.1^{myc} was indistinguishable to that of CALP4.1/CAP5.5 and co-expression of both proteins showed no obvious dominant-negative phenotype (data not shown).

Finally, both SKCRP1.6^{myc} and SKCRP1.7^{myc} displayed a staining of the whole cell body, with a clear accumulation along the plasma membrane, similar to CALP8.1^{myc} and CALP4.1/CAP5.5. However, in contrast to CALP4.1/CAP5.5 and CALP8.1^{myc}, the proteins were Triton-X100 soluble and are therefore unlikely to be cytoskeleton-associated.

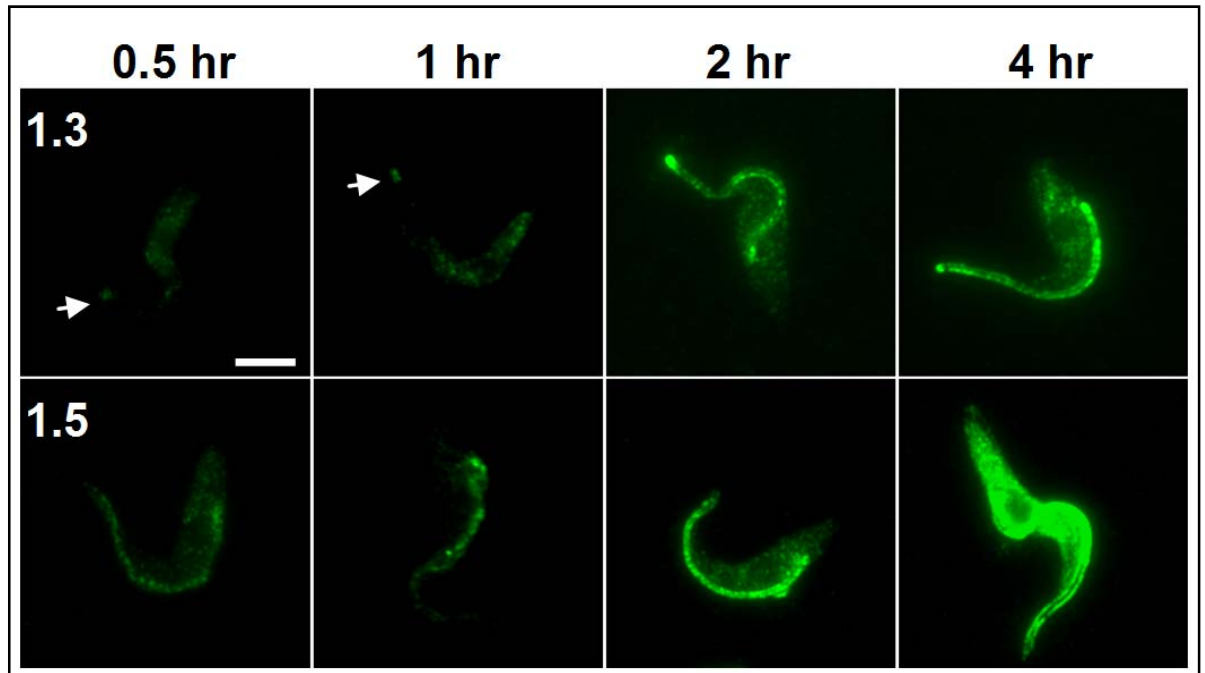
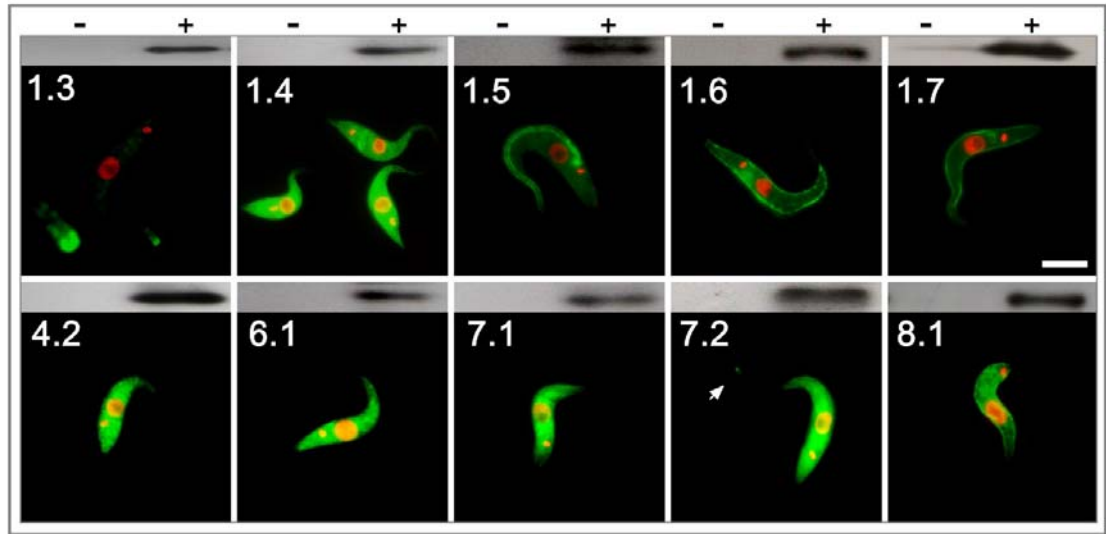


Fig.7

Immunofluorescence detection of CALP1.3&SKCRP1.5 over the indicated time using anti-cmyc antibody. Images were highly exposed due to the small amount of protein produced by the short-term induction. Note that the localization of CALP1.3 extends along the entire flagellum 2 hours post-induction and that SKCRP1.5 start to accumulate in the cell body 4 hours post-induction. Scale bar: 4 μ m

a:



b:

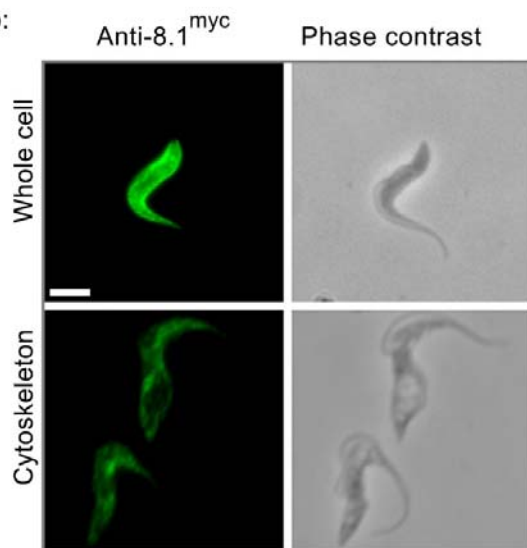


Fig.8

a. Immunofluorescence localizations of myc-tagged calpain-like proteins. The insert in the CALP1.3 image shows the enlarged flagellar tip to illustrate the cap-like structure. Note the peripheral localization of CALP1.6 and CALP1.7. An arrow indicates the signal at tip of flagellum of 7.2. Tetracycline induction experiments were shown on the top panel of each protein. Scale bar: 4 μ m

b. Immunofluorescence localization of myc-tagged CALP8.1 in whole cells and Triton X-100-extracted cells (cytoskeletons). Corresponding phase contrast images are shown on the right. Scale bar: 4 μ m

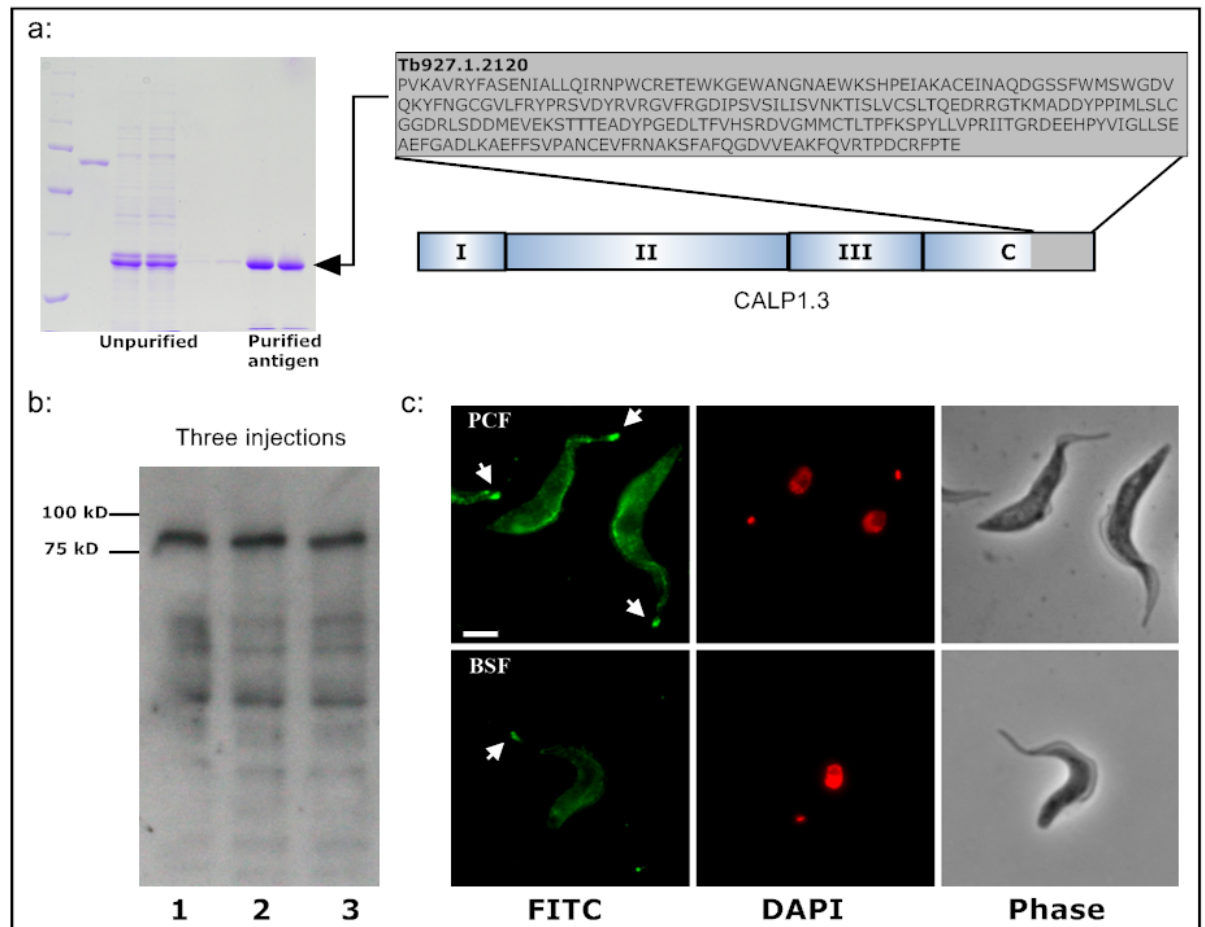


Fig.9

- a.** The 6xHis-tagged recombinant protein of CALP1.3 C-terminal region was expressed and purified on a nickel-sepharose matrix.
- b.** Sera were collected from three immunised mice and tested by western blotting. A dominant band at 80kD was recognized, corresponding to the CALP1.3 with a predicted size of 82.62kD.
- c.** Immunofluorescence localizations of CALP1.3 in procyclic(PCF) and bloodstream(BSF) form cells. Note the arrows indicating the signals at the tip of flagellum. Scale bar: 4µm

5.2.3 A C-terminal signal sequence for CALP1.3 flagellar localization

To investigate which elements of the sequence are involved in targeting CALP1.3^{myc} to the flagellar tip, a series of C-terminal deletion constructs was generated, taking into consideration the four-domain structure of the protein (Fig.10a). Deletion of 35 or more amino acids from the C-terminus of the protein led to exclusive cell body localization. The deletion of fourteen or less C-terminal amino acids had no impact on localisation in comparison to the epitope-tagged full-length protein (Fig.10b). Database searches revealed no informative hits against the C-terminal amino acids containing the putative flagellar localisation signal. Analysis of the closest relative of TbCALP1.3 in other kinetoplastids, TcCALPx.10 in *T. cruzi* (GeneDB acc. no. Tc00.1047053506563.200), revealed that both C-termini share an accumulation of basic amino acids (pI 9.7 and 10.6 for the terminal 35mer peptide in *T. brucei* and *T. cruzi*, respectively). *Leishmania* does not have a protein closely related to TbCALP1.3.

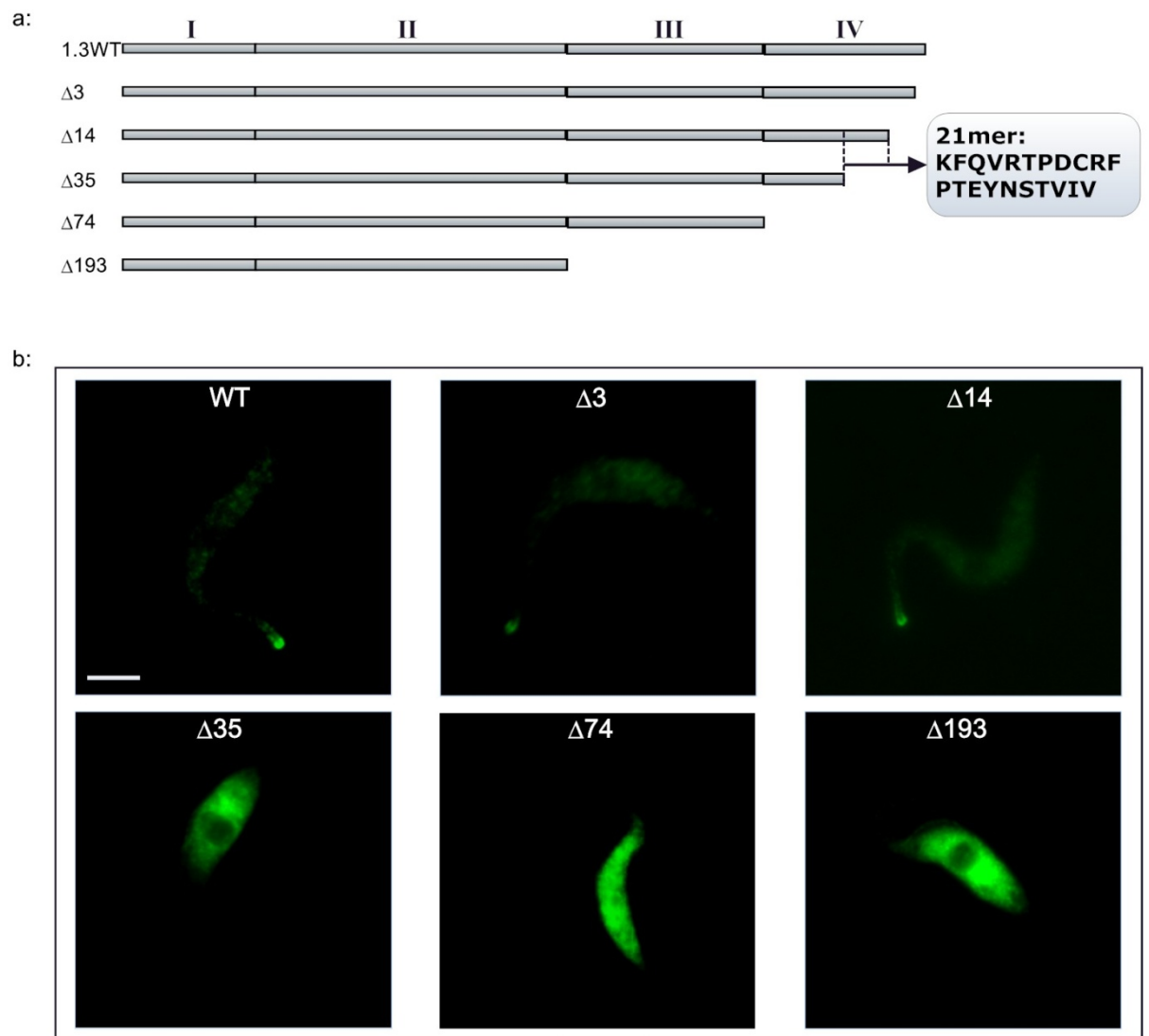


Fig.10

a. Schematic overview of the deletion constructs used to analyze targeting. 1.3WT, full length CALP1.3^{myc}; Δ193, deletion of the C-terminal 193 amino acids; Δ74, deletion of the C-terminal 74 amino acids; Δ35, deletion of the C-terminal 35 amino acids; Δ14, deletion of the C-terminal lysine rich 14 amino acids; Δ3, deletion the C-terminal three lysine. The 21mer sequence between Δ35 and Δ14 is demonstrated.

b. Immunofluorescence of CALP1.3 mutant cell lines using anti-myc antibody. Protein was mistargeted to cell body in deletion mutants Δ193, Δ77 and Δ35, but flagellar tip localization was not affected in mutants Δ14 and Δ3. The 21mer signal amino acid sequence is demonstrated in a.

5.3 Discussion

In this chapter, we have used cmyc-tagged constructs of CALP and SKCRP proteins to determine their subcellular localisation and biochemical characteristics. We identified three principle types of localisation: the flagellum, the cell body and the periphery of the cell body. CALP1.3^{myc} and probably SKCRP7.2^{myc} are localised to the tip of the flagellum and SKCRP1.5^{myc} to the entire length of the flagellum, most likely associated with the flagellar membrane. The differential flagellar localizations of CALP1.3^{myc} and SKCRP1.5^{myc} supports the view that the flagellar membrane of trypanosomes is not homogenous but compartmentalized in order to fulfill specific functions (Briggs et al., 2004). CALP1.3 and SKCRP1.5 have N-terminal dual acylation signals and it has been shown previously for *Leishmania* SMP-1 (an SKCRP-type calpain) that dual acylation is essential for flagellar localization (Tull et al., 2004). However, SKCRP 1.6, SKCRP1.7, CALP4.1/CAP5.5 and CALP8.1/CAP5.5V are also predicted or have been confirmed (CALP4.1/CAP5.5) to be dually acylated but all four proteins are not flagellar and localize to the periphery of the cell body and, in case of CALPs 4.1 and 8.1^{myc}, associate to the cytoskeleton. Therefore, there are likely to be additional signals modulating protein localisation. To test this on an acylated flagellar protein, we generated a series of C-terminal deletion mutants of CALP1.3^{myc} and demonstrated that the sequence information contained in the C-terminal 35 amino acids are essential for flagellar targeting. Also, SKCRP 7.2^{myc}, a protein without an N-terminal dual acylation signal, localises partially to the tip of the flagellum, but, in contrast to CALP1.3^{myc} and SKCRP1.5^{myc}, it is soluble after hypotonic lysis/sonication and unlikely to be associated to membranes with high affinity hydrophobic interactions. There are no sequence

similarities between SKCRP7.2 and the C-terminus of CALP1.3 and is not known how this protein is targeted to the flagellum.

Our results add to the complex data available on protein acylation and its role in protein targeting in kinetoplastids (Ginger et al., 2008, Landfear and Ignatushchenko, 2001). A well-characterised group of dually acylated flagellar proteins are calflagins (Pinto et al., 2003, Wu et al., 1992). Calflagins are flagellar calcium sensors and localize to lipid subdomains/rafts in the flagellar membrane of *T. brucei* (Fridberg et al., 2008, Tyler et al., 2009). Similar to calpain-like proteins, their flagellar localisation depends on N-terminal dual acylation. It is worth noting that Triton X-100 extractability of the flagellar calpain-like proteins was independent on whether the extraction was done at 4°C or 37°C, indicating differences to the biochemical behavior of calflagins, which are resistant to cold Triton-X100 extraction. Lipid raft-associated proteins are usually resistant to cold non-ionic detergent extraction, although exceptions have been reported (Magee and Parmryd, 2003). In *Leishmania*, the dually acylated phosphatase PPEF is targeted to the endomembrane system of the cell (Mills et al., 2007). However, when only the N-terminus, containing the dual acylation motif, is fused to GFP, the hybrid protein localised to the flagellum, indicating that the default pathway for dually acylated protein targets the flagellum. The myristoylated and palmitoylated *Leishmania* protein HASPB is secreted to the extracellular space (Stegmayer et al., 2005) and the acylated *T. cruzi* phospholipase TcPI-PLC is localised to the cell body (Furuya et al., 2000, Okura et al., 2005). The fact that not all dually acylated proteins move to the flagellum suggests a multistep targeting process for these type of proteins: the default pathway for dually acylated proteins routes to the flagellum, but additional signals redirect some

proteins to non-flagellar targets or determine the precise flagellar membrane localisation.

Four of the investigated calpain-like proteins show a homogenous localisation in the cell body. None of these proteins contains an N-terminal dual acylation motif. However, only SKCRP7.1^{myc} is soluble after hypotonic lysis/sonication, arguing against membrane-association. The other three proteins (CALP4.2^{myc}, CALP6.1^{myc} and SKCRP1.4^{myc}) are detergent-soluble but remain insoluble after hypotonic lysis/sonication. This could indicate a direct or indirect association to endomembrane systems.

SKCRP1.6^{myc} and SKCRP1.7^{myc}, containing a dual acylation motif, are also localised in the cell body, but show a clear accumulation around the periphery of the cell. This is compatible with the multistep targeting model of acylated proteins where SKCRP1.6 and SKCRP1.7 are specifically retained in association with the cell body membrane and its translocation to the flagellum is prevented.

CALP8.1^{myc}, as was shown previously for CAP5.5/CALP4.1, is associated with the subpellicular cytoskeleton. The protein is resistant to Triton X100-extraction *in vitro* and *in situ*. For both proteins, default targeting to the flagellum is somehow prevented by a direct or indirect association to the subpellicular microtubule corset probably through the N-terminal proline rich region.

6. The role of N-terminal myristoylation & palmitoylation in flagellar membrane protein trafficking

6.1 Introduction

6.1.1 The flagellum of *T.brucei*

The eukaryotic flagellum is an ancient organelle represented in most eukaryotic lineages. The basic 9+2 structure is conserved in all cases, usually assembled via the process of intraflagellar transport (Rosenbaum and Witman, 2002). *T.brucei* and related species are highly motile protozoan parasites with a single flagellum emerging from the basal body near the posterior end of the cell. The proximal end of the flagellum is embedded within a specialized membrane domain termed the flagellar pocket (for a detailed review of the flagellar pocket, see (Field and Carrington, 2009), an invagination of the plasma membrane where the entirety of endocytosis/exocytosis traffic takes place (Overath et al., 1997). The flagellum is surrounded by its own membrane containing different components to that of the plasma membrane and the flagellar pocket membrane (Tyler et al., 2009). It is attached along most of its length to the cell body, with a free distal end extending a short distance beyond the anterior end of the cell body. Apart from the obvious function in motility, the *T.brucei* flagellum is also a multifunctional organelle with critical roles in cellular morphogenesis, cell division, and potentially, sensory perception. Recent studies have revealed the *T.brucei* flagellum and flagellar motility are central to disease pathogenesis in the mammalian host and parasite development in the tsetse fly vector (Ralston and Hill, 2008). Therefore, understanding the unique

aspects of the *T.brucei* flagellum may not only contribute to the flagellum/cilium biology but also uncover the novel drug targets.

6.1.2 The cytoskeleton of *T.brucei* flagellum

The cytoskeletal structures of *T.brucei* flagellum share common features with most flagella, such as the presence of a classic 9+2 axonemal structure. It also exhibits some species-specific features (Gull, 1999). Proteomic analysis has been performed in order to address the cytoskeletal components of *T.brucei* flagellum. A *T.brucei* flagellum proteome (TbFP) consisting of 331 potential flagellar cytoskeletal proteins was generated and an *in silico* screen revealed that 208 TbFP proteins are trypanosomatid-specific and probably represent organism-specific flagellar features (Broadhead et al., 2006). One of the most distinctive features is the paraflagellar rod (PFR), a second prominent structure within the trypanosome flagellum, extending alongside the axoneme from the flagellar pocket to the flagellar tip (Vickerman, 1962). The PFR was first described 48 years ago (Vickerman, 1962, Cachon and Cosson, 1988) and consists of two main structural proteins, PFR1(PFRA) and PFR2(PFRB) (Schlaeppli et al., 1989, Deflorin et al., 1994). Depletion of either cause abnormal PFR formation and disrupted cell motility (Bastin et al., 1998, Maga et al., 1999, Santrich et al., 1997). PFR2 knockdown mutant cells are immotile with reduced flagellar beat incapable to drive cellular rotation or translocation (Branche et al., 2006, Griffiths et al., 2007). Other PFR-associated components have been recently identified. Ginger and colleagues (Ginger et al., 2005) identified two adenylate kinases, ADK-A and ADK-B, associated with the PFR. Two PFR-associated cAMP phosphodiesterases, PDEB1 and PDEB2,

have been characterized and shown to affect parasite virulence (Oberholzer et al., 2007). A recent systematic study using quantitative mass spectrometry and two-dimensional differential in-gel electrophoresis (2D-DIGE) has identified 20 novel PFR-associated proteins by comparing protein profiles of wild-type and PFR2 knockdown mutants (Portman et al., 2009). Seven out of 20 have been epitope tagged and all showed PFR localization (Portman et al., 2009). Overall, the recent studies indicate that PFR is not only a passive structural support to the axoneme, but also may serve as a scaffold for the assembly of regulatory and metabolic proteins required for flagellar function.

Another unique feature of *T.brucei* flagellum is the lateral attachment to the cell body along the flagellum attachment zone (FAZ) (Gull, 1999, Kohl and Gull, 1998, Sherwin and Gull, 1989, Vickerman, 1962, Vickerman, 1969a), which is defined by a cytoplasmic, electron-dense filament of mostly unknown composition and by four microtubules of the subpellicular corset that are associated with an unknown membranous compartment (Vickerman, 1969a). A network of filaments links the FAZ to both the axoneme and the PFR in the flagellum. Very few FAZ proteins in *T.brucei* have been identified. Flagellum adhesion glycoprotein 1 (Fla 1) localizes to the FAZ and is required for flagellum attachment (LaCount et al., 2002). Another FAZ component, FAZ1, was recently identified by screening an expression library with the monoclonal antibody L3B2 that recognizes the FAZ filament (Vaughan et al., 2008). Knockdown of FAZ1 results in abnormal FAZ assembly and defects in flagellar attachment (Vaughan et al., 2008). Notably, flagellar assembly is not blocked in the absence of attachment via the FAZ to the cell body (Vaughan et al., 2008).

6.1.3 The flagellar membrane and matrix

Depending on the different composition and function, the surface membrane of a trypanosome is divided into three distinct but contiguous domains: the pellicular (cell body) membrane; flagellar pocket membrane and flagellar membrane (Gull, 2003). Although the flagellar membrane represents a critical interface between the parasite and its hosts, the flagellar membrane components and the soluble flagellar matrix in *T.brucei* still represents largely uncharted territory (Webster and Russell, 1993). Eukaryotic flagella have been viewed as a sensory organelle that receive and transduce signals from the external environment (Eisenbach and Giojalas, 2006, Zhang and Snell, 1994), but a sensory function of the *T.brucei* flagellum has not yet been directly demonstrated. An increasing number of flagellar membrane proteins have been identified during the past decade, as well as the existence of the IFT machinery responsible for flagellar assembly (Absalon et al., 2008).

6.1.3.1 Intraflagellar transport(IFT)

Intraflagellar transport (IFT) is a flagella-specific motility process that was initially discovered in the biflagellate green alga *Chlamydomonas reinhardtii* (Kozminski et al., 1993), and found to be conserved within all eukaryotic organisms where this process has been studied, including kinetoplastids (Rosenbaum, 2002, Kohl et al., 2003). IFT is a microtubule-dependent particle motility that moves the necessary flagellar protein components from the cell body into and out of the flagellum (Rosenbaum and Witman, 2002). To enter the flagellum, the proteins are carried by a flagellar-bound IFT protein complex. It is then transported from the base to the tip of the flagellum along the

flagellar microtubules by kinesin II motor proteins (Rosenbaum and Witman, 2002). At the tip of the flagellum, the IFT particles reverse direction and are transported back to the base by cytoplasmic dynein 1b, where the IFT particles and motors are recycled. An intact IFT system is important for the formation of cilia and flagella, and a number of other processes (Rosenbaum and Witman, 2002, Scholey, 2003). Defects in IFT lead to failing in the assembly of motile flagella/cilia in *Chlamydomonas reinhardtii* (Pazour et al., 2000), sea urchin (Morris and Scholey, 1997), *Tetrahymena thermophila* (Brown et al., 1999), *Caenorhabditis elegans* (Perkins et al., 1986, Signor et al., 1999, Qin et al., 2001) and mice (Marszalek et al., 2000, Pazour et al., 2002). In *T.brucei*, several conserved IFT components have been identified and shown to function mainly in flagellar biogenesis (Kohl et al., 2003, Davidge et al., 2006, Absalon et al., 2007, Absalon et al., 2008). Interestingly, it seems that the growth and extension of the flagellar membrane can still take place in the absence of IFT (Absalon et al., 2008).

6.1.3.2 Lipid rafts in the flagellar membrane

Previous studies have shown that the trypanosome pellicular membrane and flagellar membrane have distinct lipid compositions (Tetley, 1986). Specifically, the lipid rafts, were found to be enriched in the *T.brucei* flagellar membrane (Emmer et al., 2009, Fridberg et al., 2008, Tyler et al., 2009). Lipid rafts are heterogeneous specialized membrane microdomains enriched in cholesterol and sphingolipids that play crucial roles in processes such as cell signaling, protein sorting and lipid trafficking in polarized cells (Pike, 2003). These tightly packed molecules form small platforms within the membrane and are thought to constitute the ‘liquid ordered’ areas, which makes them

more resistant to nonionic detergents such as Triton X-100 (Fridberg et al., 2007). Lipid rafts often act as pre-platforms for correct targeting and signal transduction. However, a fluorescence-resonance energy transfer (FRET) analysis was used to test whether the raft-associated proteins are concentrated with lipid rafts in T cells, and showed that they are instead randomly distributed (Glebov and Nichols, 2004). Measurements of protein diffusion by fluorescence-correlation spectroscopy (FCS) also indicated that GPI-anchored proteins undergo transient confinements in isolated microdomains (Lenne et al., 2006). Such findings suggest that lipid rafts are probably small and dynamical structures, and evoke mechanisms by which cells organize and stabilize these small domains in an ‘as-needed’ basis (Kenworthy, 2008, Viola and Gupta, 2007). Nevertheless, a number of proteins, especially those that are dually acylated, GPI-linked, or transmembrane, are typically recruited into rafts (Fridberg et al., 2007). For dually acylated proteins, fatty acid length can influence the association with lipid rafts. A myristoylate group (C14, saturated) cannot provide sufficient affinity to the lipid rafts, but the palmitoylation (C16, saturated) is sufficient for stable association (Benting et al., 1999, Melkonian et al., 1999).

6.1.3.3 Palmitoylation-mediated targeting

Soluble proteins can be anchored to membranes by adding lipids, such as myristoyl groups (C14,saturated) (Towler et al., 1987) or isoprenyl groups (C15 or C20) (Reiss et al., 1990) (Fig.11). These modifications are irreversible and essential for membrane targeting. In contrast, the thioester linkage of palmitic acid (C16, saturated) to cysteine residues (S-palmitoylation) is a reversible modification catalyzed by membrane-bound

palmitoyl transferases (PATs), which can occur shortly after protein synthesis or at later stages throughout the protein life-time.

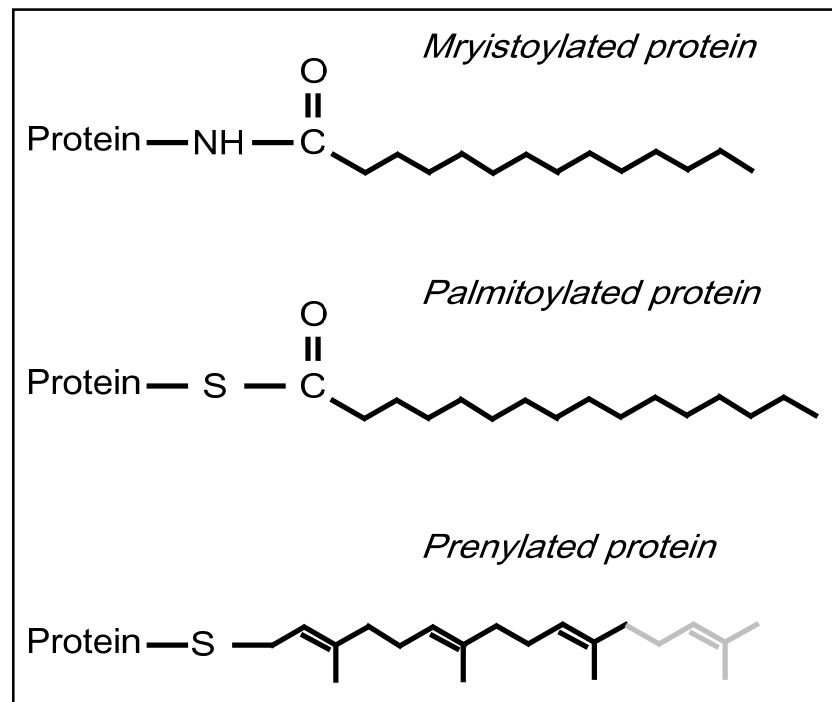


Fig.11 Demonstration of three types of protein lipid modification

Palmitoylation can provide a stable membrane association that is about 5 times stronger than that provided by a single geranylgeranyl group, 100 times stronger than that provided by a single farnesyl group and 10 times stronger than that provided by a single N-myristoyl moiety (Shahinian and Silviu, 1995). Therefore, palmitoylation can be viewed as a secondary signal for membrane association, as the primary acyl modification (e.g. with myristic acid) directs the protein to a membrane that brings the protein in the proximity of PAT enzymes. In some cases, palmitoylation subsequently stabilizes the membrane association of proteins that have arrived at a membrane compartment via weak or transient interactions, such as those mediated by N-myristoylation. Palmitoylation also occurs on some transmembrane proteins that are already tightly associated with membranes. For examples, approximately 80% of

G-protein coupled receptors (GPCRs) undergo C-terminal palmitoylations (Escriba et al., 2007). Apart from facilitating membrane association, the fact that palmitoylation in GPCRs is very dynamic (Magee et al., 1987, Skene and Virag, 1989) suggests it is likely to be involved in a regulatory mechanism in the cell (Mumby, 1997). Depletion of C-terminal palmitoylation sites in human proteinase-activated receptor 1 (PAR1), a subfamily of GPCRs, does not reduce the membrane bound strength dramatically, but affects the downstream Ca^{2+} signaling as well as the ERK1/2 pathways (Dan, PhD thesis).

In kinetoplastids, no regulatory role of protein palmitoylation has been demonstrated. However, several studies have revealed that palmitoylation is important in flagellar membrane protein localizations (Godsel and Engman, 1999, Tull et al., 2004, Emmer et al., 2009). Therefore, the N-terminal dual acylation is believed to be one of the flagellar membrane targeting determinants. As demonstrated in chapter 5, CALP1.3 and SKCRP1.5 were both localized in the flagellar membrane and harbor N-terminal acylation motifs. To determine the potential role of palmitoylation in flagellar membrane targeting, we have performed a mutagenesis analysis on both proteins and propose a model of flagellar membrane protein trafficking.

6.2 Results

6.2.1 CALP1.3 & SKCRP1.5 are palmitoylated *in vivo*

Both CALP1.3 and SKCRP1.5 have predicted N-terminal dual acylation motifs. The acyl-biotin exchange assay was used to confirm the palmitoylation. As described in chapter 3, in the hydroxylamine-labeled sample, only the palmitic acid is replaced with biotin and can be subsequently purified using avidin-coated beads. The same c-myc tagged cell lines that were used for localisation studies (see Chapter 4) were used for this biochemical assay (described in Chapter 3.7). CALP1.3^{myc} and SKCRP1.5^{myc} were purified and subsequently detected using anti-cmyc antibody. BiP (kindly provided by J. Bangs) and CALP4.1/CAP5.5 were used as controls (Hertz-Fowler et al., 2001).

Both CALP1.3 and SKCRP1.5 are palmitoylated *in vivo* (Fig.12). The presence of bands in the hydroxylamine-labeled eluate fraction indicated the palmitoylation was accomplished and not affected by the C-terminal epitope tag. Surprisingly, SKCRP1.5 displayed three bands after purification. Experimental artifacts such as unspecific bindings of antibody were excluded when SKCRP1.5 was compared with other c-myc tagged calpain-like proteins using long exposure time. The over-exposure did not reveal same bands in other proteins (data not shown). Therefore, the presence of this multiple band pattern is specifically associated with the purification of SKCRP1.5^{myc}. The reason of why SKCRP1.5^{myc} is showing triple bands is still unclear. In the acyl-biotin exchange assay, the triple bands were only observed in the hydroxylamine-labeled eluate fraction. One explanation is probably due to the sample loading amount, as the eluate fraction purified by avidin-coated beads is specifically concentrated with palmitoylated proteins, whereas samples are too diluted in the other fractions to be detected.

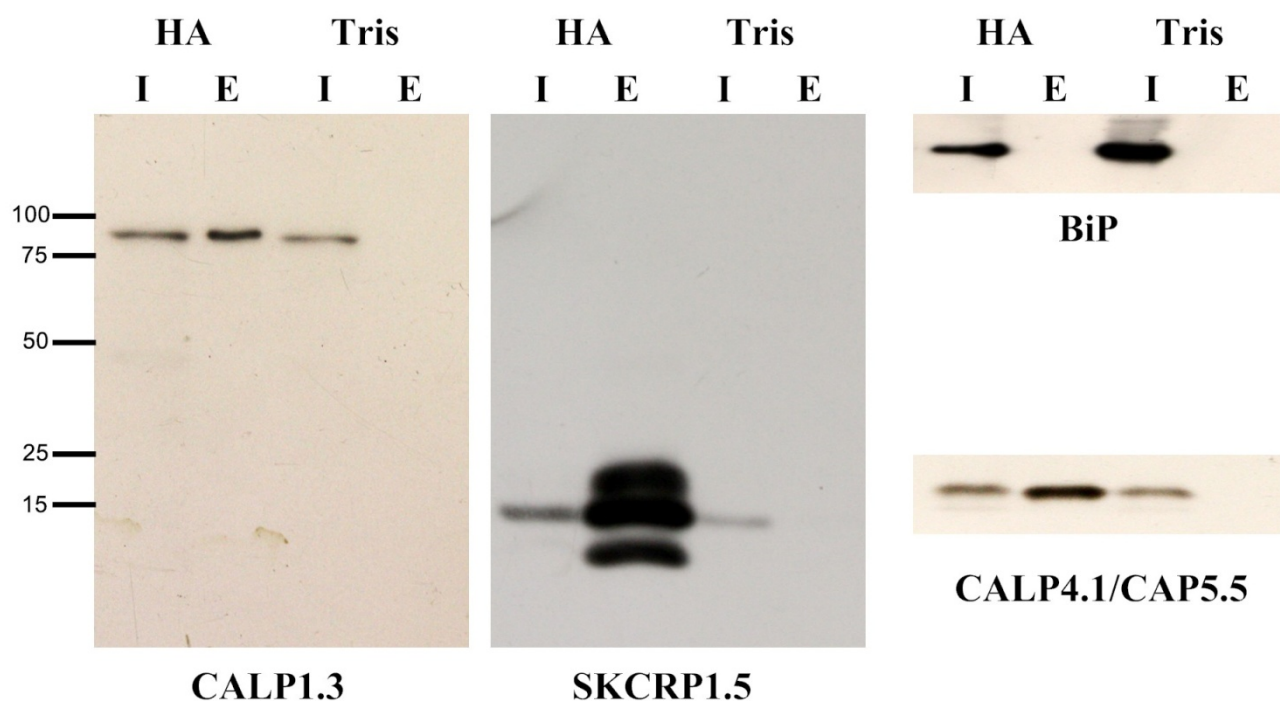


Fig.12 Myc-tagged CALP1.3 and SKCRP1.5 are palmitoylated in vivo.

Using an acyl-biotin exchange reaction only in the hydroxylamine-treated sample palmitic acid is replaced with biotin and can subsequently be purified using avidin-coated beads, as described in Materials and Methods. Therefore, detection with a suitable antibody in the purified eluate fraction (E) indicates palmitoylation. Samples treated with Tris-buffer instead are not deacylated and cannot be biotinylated and purified. Samples from input (I) and eluate (E) fractions were analyzed by western blot using anti-myc antibody. As controls were used CAP5.5, known to be palmitoylated and detected with anti-CAP5.5 monoclonal antibody, and BiP, which is not palmitoylated and was detected with polyclonal anti-BiP antibody (kindly provided by J. Bangs).

6.2.2 The N-terminal acylation motif is required for flagellar targeting

A site-directed mutagenesis study was performed on the N-terminus of CALP1.3 and SKCRP1.5 to investigate whether the N-terminal dual acylation is responsible for the flagellar targeting. The c-myc tagged expression system (described in Chapter 3.8) was used to generate deletion mutants with either the glycine in position 2 ($\Delta G2$), the cysteine in position 3 ($\Delta C3$) or both residues ($\Delta G2C3$) deleted. Immunofluorescence studies revealed the wildtype (WT) protein to be in the flagellum. All of the mutant proteins were mis-targeted and found diffusely throughout the cytoplasm, indicating that both myristoylation and palmitoylation are important for flagellar localization (Fig.13a). However, the different wildtype localizations of CALP1.3 and SKCRP1.5 (flagellar tip and entire flagellum, respectively) clearly suggests that the dual acylation motifs do not account for the protein targeting to the precise sub-flagellar localization and additional signals are likely to exist. On the other hand, protein acylation is important to facilitate the association with cellular membranes (Magee and Seabra, 2005). I have confirmed the importance of acylation for membrane-association by showing that the N-terminal acylation depletion mutants are partially solubilised after hypotonic/sonication lysis, indicating the protein-membrane association has been significantly reduced whereas the wildtype of CALP1.3 and SKCRP1.5 are resistant to solubilisation (Fig.13b).

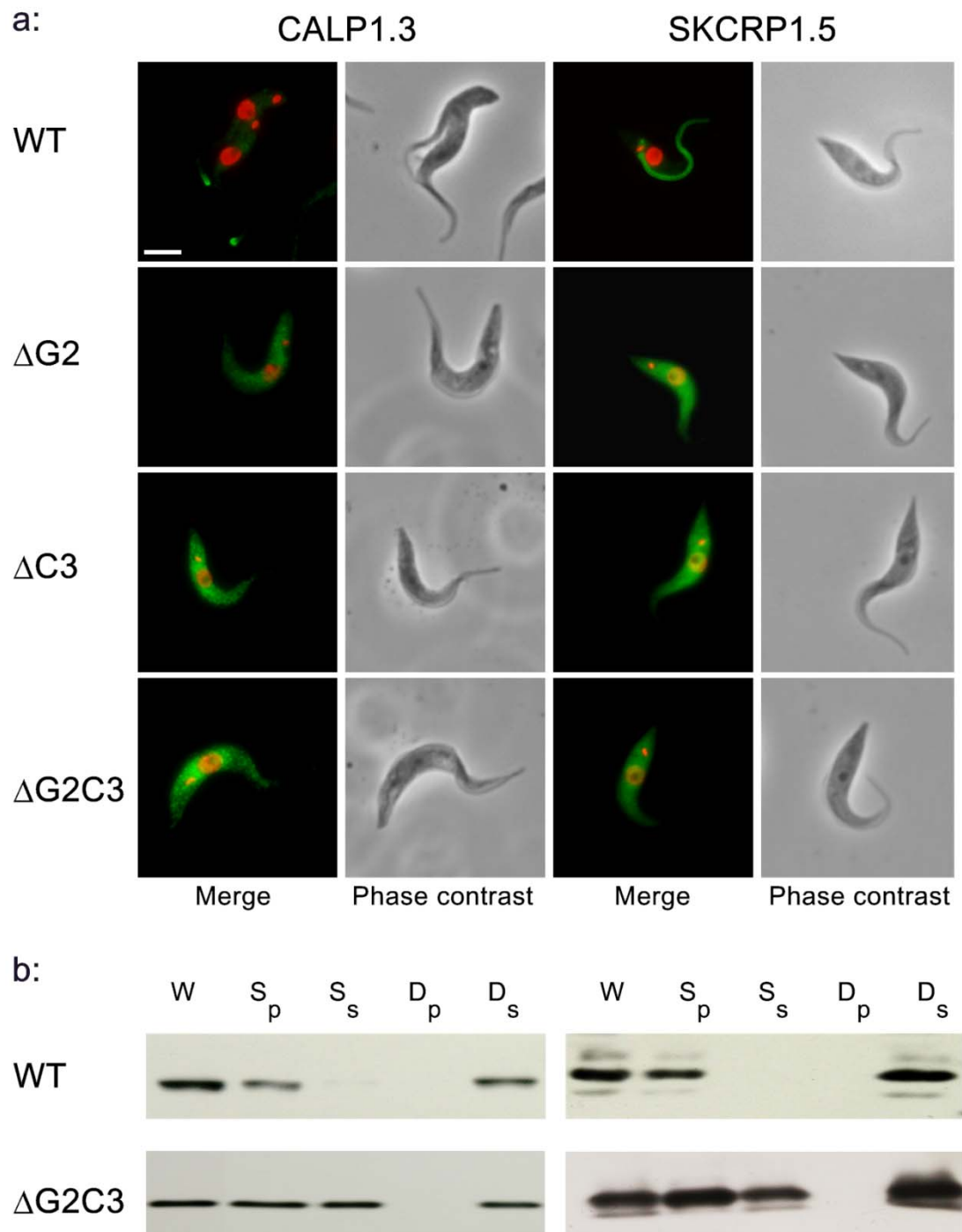


Fig.13

a. Immunofluorescence of CALP1.3 and SKCRP1.5 mutant cell lines using anti-myc antibody. Both were mistargeted to cell body in deletion of N-terminal acylation motif. WT: wild type; Δ G2: the Glycine2 deletion; Δ C3: the Cystein3 deletion; Δ G2C3: the Glycine2Cystein3 deletion. Scale bar: 4 μ m

b. Cell fractionation analysis of Δ G2C3 mutant cell lines. Analysis was performed as described previously(Chapter II). Additional proportions in the S_s fraction indicated the compromised membrane association. Whole cell extracts (W), sonication-insoluble fraction (S_p), sonication-soluble fraction (S_s), detergent-insoluble (D_p) and detergent-soluble fraction(D_s)

6.2.3 N-terminal palmitoylation is not the only flagellar targeting determinant

As the N-terminal acylation is important for the flagellar localization of CALP1.3 and SKCRP1.5, I then asked if it is the signal directing proteins to the flagellar membrane. To test this, we have fused the N-terminal 30 amino acids of CALP1.3 and SKCRP1.5 with GFP, respectively. The palmitoylation were confirmed by acyl-biotin exchange assay as described previously (Fig.14a, Fig.15a). Full length CALP1.3 and SKCRP1.5 fused with GFP were generated as controls.

1.3WT_GFP (CALP1.3 wildtype followed by GFP) was localized along the entire flagellum membrane (Fig.14b). Overexpression of CALP1.3^{myc} exhibited a similar mis-localization (Fig.7, chapter 5). But for 1.3WT_GFP, the flagellar tip localization was not observed even at early time point (1 hr after induction). The localisation of 1.3N_GFP (CALP1.3 N-terminal 30 amino acids followed by GFP) was indistinguishable from 1.3WT_GFP, along the entire flagellar membrane, suggesting the first 30aa region with N-terminal acylation is sufficient for flagellar targeting (Fig.14b). However, due to the absence of flagellar tip localization in both 1.3N_GFP and control (1.3WT_GFP), it cannot be concluded that the N-terminus is specifically required for the tip localization. Interestingly, when data were collected 2 hr after induction, an additional signal was observed in 1.3N_GFP mutant cell lines, representing a single dot at one side of the flagellum, often at the connection point between the flagellum and the anterior end of the cell body. The additional signal disappeared after 8 hr or longer induction time (Data not shown). A similar phenomenon was not observed in the 1.3WT_GFP cell line.

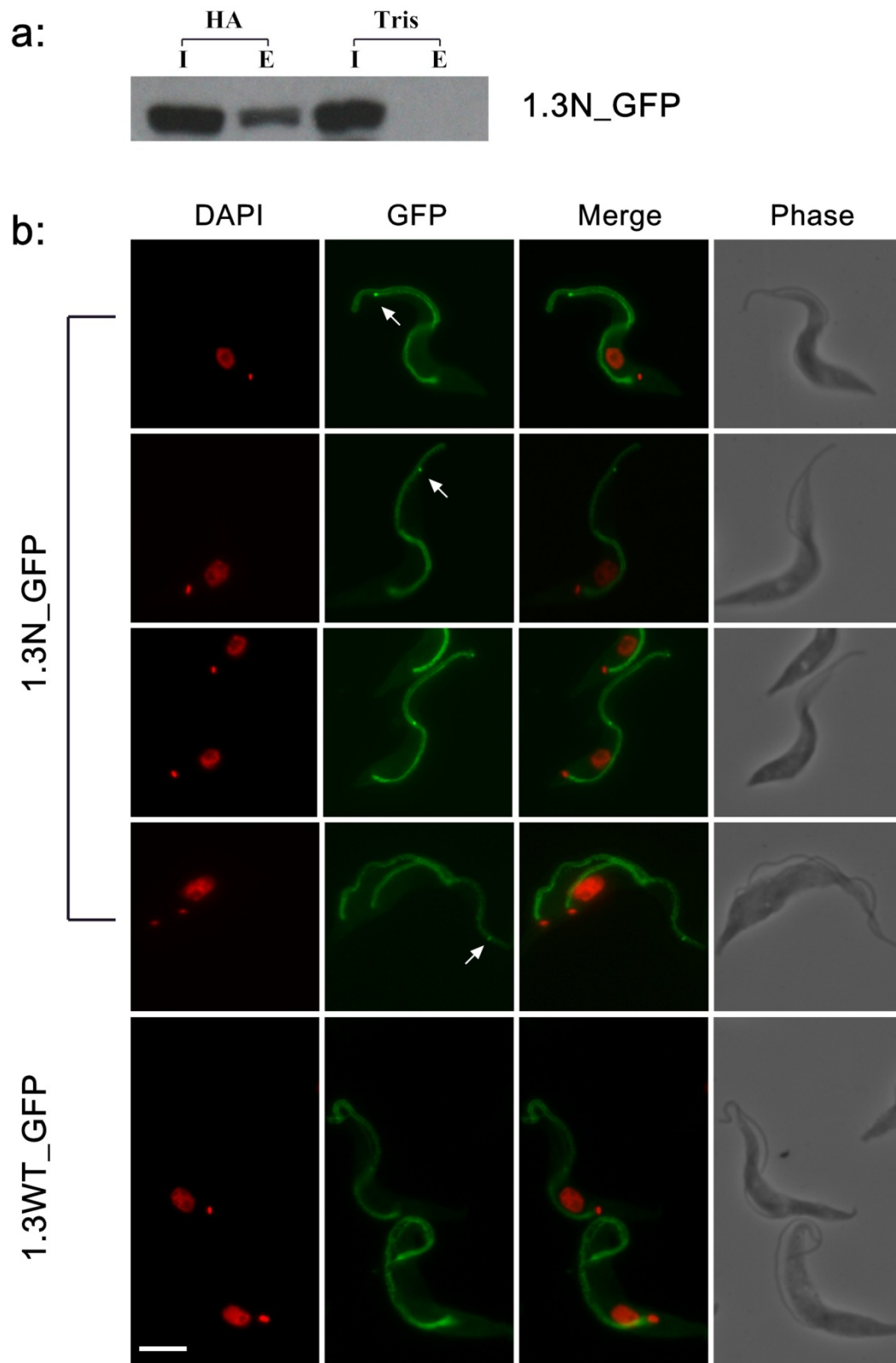


Fig.14

a. The hybrid protein CALP1.3 30aa N terminus followed by GFP(1.3N_GFP) is palmitoylated *in vivo*. Acyl-biotin exchange assay was performed as described in Materials and Methods

b. Immunofluorescence of 1.3_GFP wild type and mutant cell lines. Data were collected 2-3 hours after induction. Full length CALP1.3 followed by GFP (1.3WT_GFP) localised at the entire flagellum (bottom panel) as well as the 1.3N_GFP. Note the additional dot signals unique to 1.3N_GFP(arrow). Scale bar: 4 μ m

1.5WT_GFP (SKCRP1.5 wild type followed by GFP) showed the same flagellar membrane localization as the cmc-tagged protein (Fig.15b). The 1.5N_GFP (SKCRP1.5 N-terminal 30 amino acids followed by GFP) mutant is excluded from the flagellum, but signals are now enriched around the perimeter of the cell body (Fig.15b). Therefore, although the 1.5N_GFP is palmitoylated, its N-terminal 30 amino acids are not sufficient for flagellar targeting. Furthermore, over-expression of 1.5N_GFP fusion protein seems to destabilize cells during the standard immunofluorescence microscopy fixation process. Burst cells were frequently observed compared to those expressing 1.5WT_GFP or any other GFP fusion proteins. 1.5N_GFP in burst cells appeared to be not associated to the plasma membrane, but exhibited a cytoskeletal association (Fig.16). The destabilization was only observed during the fixation process and there was no growth defect or observable abnormal phenotype in the 1.5N_GFP cell line. Overall, our existing data suggest that although the N-terminal myristoylation/palmitoylation is a necessary step in targeting proteins to flagellar membrane, but is not sufficient to achieve this.

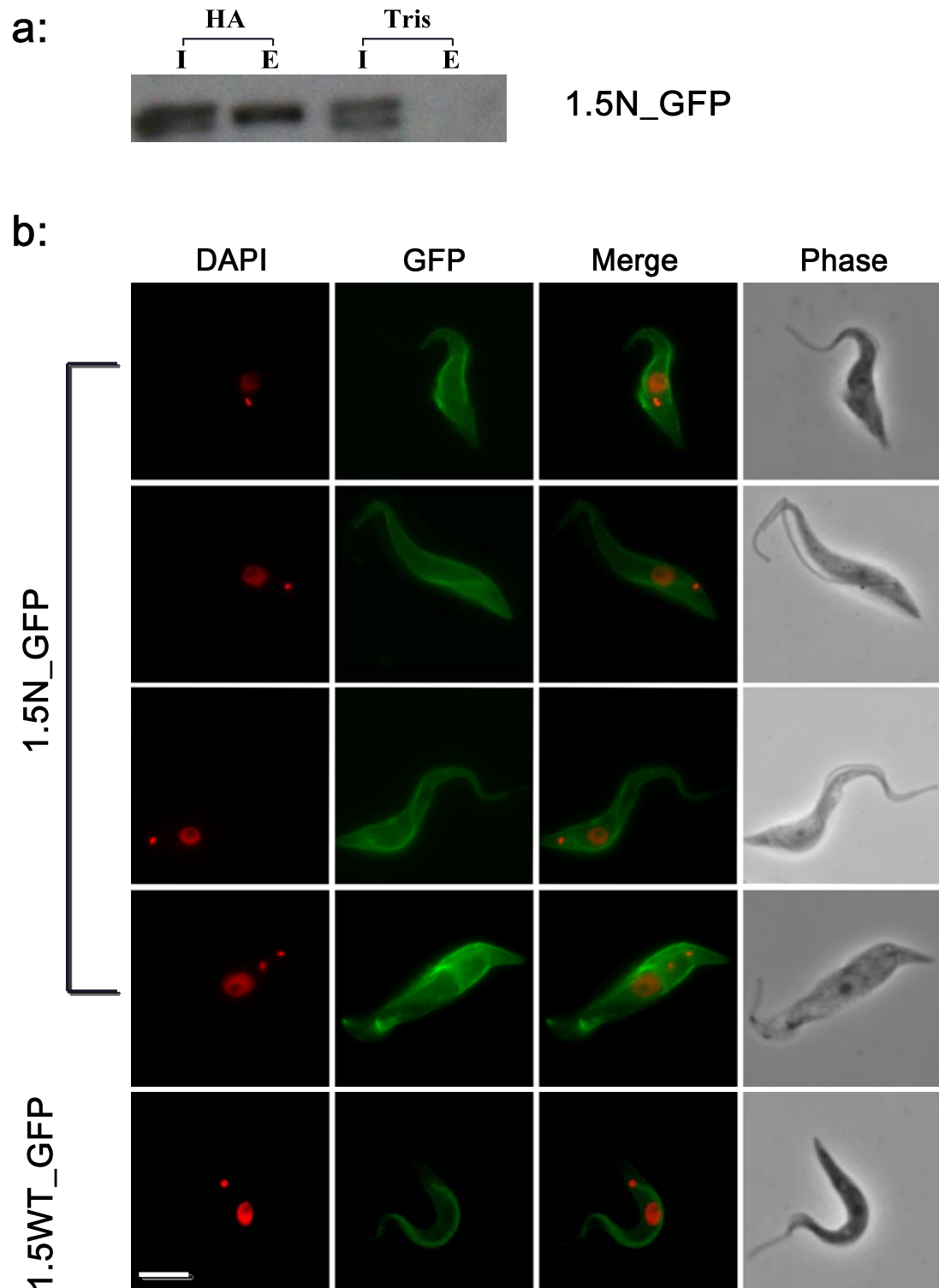


Fig.15

a. The hybrid protein SKCRP1.5 30aa N terminus followed by GFP(1.5N_GFP) is palmitoylated *in vivo*. Acyl-biotin exchange assay was performed as described in Materials and Methods

b. Immunolocalization of 1.5_GFP wild type and mutant cell lines. Full length SKCRP1.5 followed by GFP(1.5WT_GFP) localised at the entire flagellum (bottom panel), where as 1.5N_GFP concentrated around the perimeter of the cell body. Scale bar: 4μm

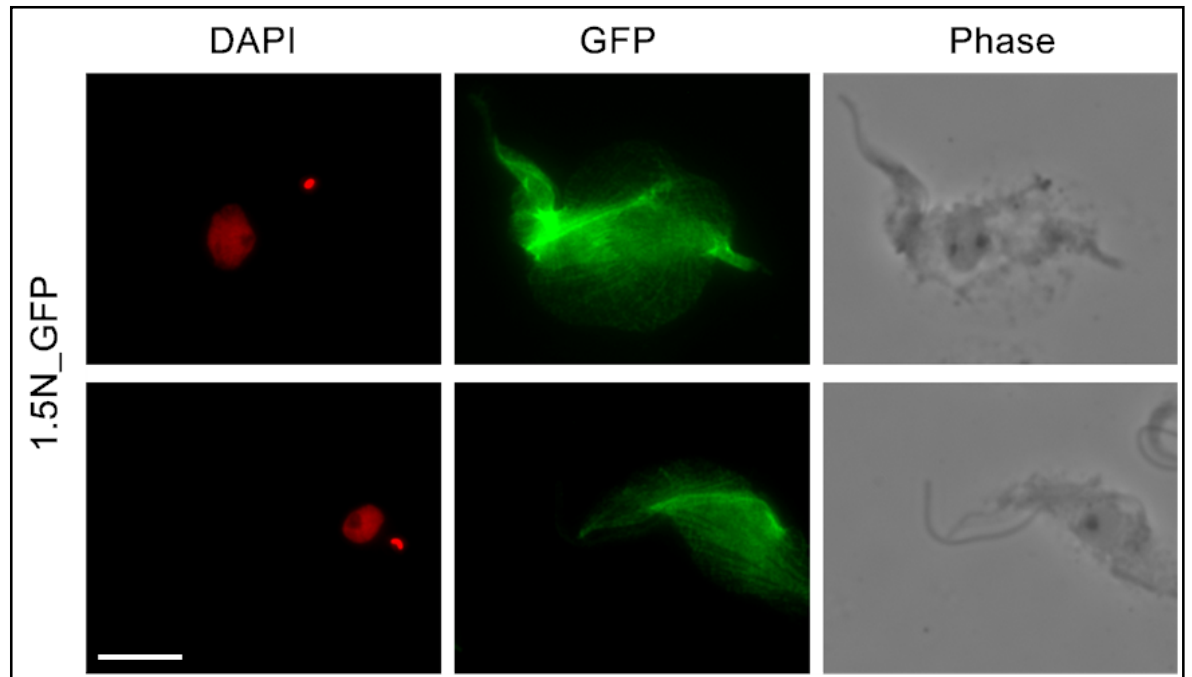


Fig.16 Immunofluorescence localizations of the 'burst' 1.5N_GFP cells. Cells were not pre-treated with detergent-containing buffers. Note the signals along the cytoskeletal microtubules. The right panel shows the corresponding phase-contrast images. Scale bar: 4 μ m

6.3 Discussion

6.3.1 Dual acylation is required for crossing the selective barrier of the flagellum

Dual acylation plays an important role in sorting proteins to the cellular membranes. Here, our data have shown that both myristoylation and palmitoylation are required for targeting CALP1.3 and SKCRP1.5 to the flagellar membrane in *T.brucei*. Depletion of their N-terminal acylation motifs compromised the membrane association and resulted in cell body mis-localization.

Shahinian and colleagues have proposed a “kinetic bilayer trapping” model for the trafficking of acylated membrane proteins. Proteins with a single lipophilic group (myristoyl or farnesyl) transiently interact with intracellular membranes. The single acylated protein that reaches the membrane will be rapidly palmitoylated by a membrane bound palmitoyl transferase (PAT) (Shahinian and Silvius, 1995). In such cases, the key function of the palmitoylation is to serve as a membrane anchor that increases relative membrane affinity, which is important for stable membrane association. This enzyme-dependent model has been applied to explain the targeting mechanism of some well studied dual acylated proteins such as the mammalian H- and N-Ras. The primary signal for membrane association of these proteins is the C-terminal farnesylation, which usually occurs on the cysteine residue of a conserved CAAX motif (C is Cysteine, AA are two Aliphatic residues and X represents any C-terminal amino acid depending on different substrate specificity). The CAAX motif predominantly directs proteins to the ER and Golgi membranes but provides only a weak affinity (Peitzsch and McLaughlin, 1993, Shahinian and Silvius, 1995, Choy et al., 1999). Using fluorescence recovery after photobleaching (FRAP), the single farnesylated Ras was

found to undergo rapid diffusional exchange between the cytosol and endomembranes (Goodwin et al., 2005, Rocks et al., 2005). In contrast, dual lipid modifications can provide a relatively stable membrane association. The subsequent palmitoylation of Ras increased the strength of membrane interaction (Magee et al., 1987), and redirected Ras to the plasma membrane. An acylation model of H-Ras and N-Ras in the mammalian cells has been proposed where a single farnesylated Ras may be palmitoylated by a putative PAT on the ER membrane and traffics to the Golgi/plasma membrane via the vesicle-mediated classic secretory pathway. Alternatively, farnesylated Ras may transiently interact with the Golgi via non-vesicular exchange and subsequently be palmitoylated on the Golgi membrane by another putative PAT (Linder and Deschenes, 2007).

Another group of proteins which may undergo the “kinetic bilayer trapping” mechanism are N-terminal dual acylated membrane proteins (e.g. CALP1.3 and SKCRP1.5). The primary signal for initial membrane association is the N-myristoylation, which refers to the co-translational linkage of myristic acid (C14, saturated) to the N-terminal glycine residues (Wilcox et al., 1987). Subsequent palmitoylation often occurs on the cysteine residues that are adjacent to or near an N-myristoylated glycine residue (Resh, 1999). The N-myristoylation is also believed to be a prerequisite for palmitoylation. Mutagenesis studies of the N-terminal dually acylated protein p59fyn (a member of Src family) revealed that the palmitoylation was prevented when glycine 2 was mutated to alanine (Alland et al., 1994). For the same reason, the glycine 2 depleted mutants (Δ G2) in this study, were unlikely to be palmitoylated due to the absence of N-myristoylation. According to the “kinetic bilayer trapping” model, proteins with a single myristoylation

may frequently interact with multiple cellular membranes, and are stabilized by the subsequent palmitoylation. The single myristoylated CALP1.3 and SKCRP1.5 ($\Delta C3$ mutants) were retained in the cell body, but excluded from the flagellum. Over-expression of the protein (>16 hours) did not force the proteins to the flagellum but simply further accumulated them in the cell body. When both acylation steps were prevented ($\Delta G2C3$ and probably $\Delta G2$), the same phenomenon was observed. None of the mutants was able to spread into the flagellum, suggesting the flagellar microenvironment is a highly restrictive area. Instead of entering the flagellum through passive diffusion, proteins are transported by regulated delivery systems (e.g. IFT system) (Blacque et al., 2008).

In contrast to the H- and N-Ras proteins, which harbor a conserved CAAX motif as the ER/Golgi retention signal, the identifiable consensus sequence for the dual acylation proteins is the Met-Gly-Cys motif at their N-termini, and a conserved Ser/Thr residue near the Gly which is necessary for the N-myristoylation. The downstream 10-15 amino acids are believed to be important for the subsequent palmitoylation (Resh, 1999) but no strictly conserved motifs have been identified. In the calpain-like protein family, it has been shown that dually acylated proteins with the conserved N-terminal Met-Gly-Cys motif can still exhibit various subcellular localizations, ranging from the tip of flagellum (CALP1.3), the entire flagellar membrane (SKCRP1.5), but importantly also outside of the flagellum, such as to the subpellicular membrane (SKCRP1.6, SKCRP1.7) and the subpellicular cytoskeleton (CALP4.1/CAP5.5, CALP8.1/CAP5.5V) (Hertz-Fowler et al., 2001). For this reason, instead of concluding that the N-terminal acylation is a flagellar targeting signal, an alternative explanation, congruent with our and published data, is

probably the existence of a 'flagellar diffusion barrier', which is blocking the transient interaction of the single-myristoylated or non-acylated proteins with the flagellar membrane, preventing the passive entry into the flagellum. A potential candidate for such a 'barrier' has been previously observed as a cytoskeletal 'neck' region within the flagellar pocket, termed the flagellar pore complex (FPC). It contains the transition fibre structures that extends from the distal end of the basal body and connects each of the nine basal-body triplet microtubules to the flagellar membrane (Rosenbaum and Witman, 2002, Gull, 2003). The FPC is believed to act as a gateway, providing a selective barrier for protein entry into the flagellum (Dentler and Adams, 1992, Fridberg et al., 2007). Previous studies of dually acylated flagellar membrane proteins also support this view. Two dually acylated proteins, calflagin and SMP-1 localized to the flagellar membrane of *T.brucei* and *L.major*, respectively. The depalmitoylated mutants were mis-targeted to the cell body, unable to enter the flagellum (Emmer et al., 2009, Tull et al., 2004). Therefore, although it cannot be concluded that the N-terminal acylation is the actual flagellar targeting signal, the completion of myristoylation/palmitoylation appears to be one of the essential requirements for passing through the selective barrier of the flagellum.

6.3.2 Dual acylation is an important early step in flagellar protein targeting

Previously, we have suggested a multistep targeting process that the dually acylated proteins can be relocated to non-flagellar targets by the additional signals (Liu et al., 2009). The additional data presented here show that the dually acylated 1.5N_GFP fusion protein (SKCRP1.5 N-terminal 30aa fused with GFP) was targeted to the cell

body and associated with the cytoskeleton, indicating the N-terminal Met-Gly-Cys acylation motif is not the actual flagellar targeting element. Therefore, additional signals are still required not only for the non-flagellar dually acylated proteins, but also for those that are destined for the flagellum. Furthermore, depletion/mutation of protein N-terminal acylation often results in non-specific homogeneous cell body localization (e.g. cytosol), whereas the GFP fusions containing the dually acylated N terminus preferably exhibit organelle-specific localization, that are either similar to the wild type or have an unrelated specific localization (**Table 2**). Collectively, these data support the view of a multistep targeting process and further imply that the dual acylation is necessary in a chain of events that defines localization specificity, but that the precise destination is determined downstream by other signaling elements within the protein. Therefore, a step operating as the “decision point” is likely to exist, and the dual acylation, instead of directing proteins to specific positions, serves as the prerequisite for final decision steps.

Name	Wild Type	N-terminus + GFP	Δ myr/palm	Δ palm	Ref
CALP1.3	Flagellar tip	Flagellar membrane	Cytosol	Cytosol	This study
SKCRP1.5	Flagellar membrane	Cytoskeleton	Cytosol	Cytosol	This study
Calflagin	Flagellar membrane		Cytosol	Plasma membrane	(Tyler et al., 2009)
SMP-1	Flagellar membrane		Cytosol	Cytosol	(Tull et al., 2004)
PPEF	Endocytic system	Flagellum	Cytosol	Cytosol	(Mills et al., 2007)
HASPB	Plasma membrane	Plasma membrane	Cytosol	Cytosol	(Stegmayer et al., 2005)
TcPI-PLC	Plasma membrane		Cytosol		(Okura et al., 2005)
α 19-Giardin	Flagellum		Cytosol		(Saric et al., 2009)
PDE2A3	Plasma membrane		Cytosol	ER/Golgi	(Russwurm et al., 2009)

Table.2 A number of dually acylated proteins that have been studied. Elimination of N-terminal acylation often results in non-specific cytosolic localizations. When the acylated N-terminus were fused with GFP, the specific localizations were observed. Δ palm, elimination of the palmitoylation, Δ myr/palm, elimination of both myristoylation and palmitoylation

6.3.3 A model for the delivery of flagellar/ciliary membrane proteins

The ciliary (refers to both flagella and cilia as they are not fundamentally different) microenvironment and the cytosol/plasma membrane are separated by a selective barrier, suggesting the existence of certain trafficking systems for the delivery of flagellar proteins. However, the basic mechanisms are still unclear. Polarized exocytosis to the base of the cilium has been the prevailing model for the transportation of ciliary membrane proteins (Emmer et al., 2010). The ultrastructural studies revealed that vesicles containing ciliary membrane protein fused with the periciliary membrane in algae (Bouck, 1971). Subsequent experiments revealed that vesicles were specifically targeted and accumulated at the base of the connecting cilium before entry (Papermaster et al., 1985). An immunogold electron microscopy study revealed the IFT-particle proteins are also docked at the base (transition fibre) of the flagellum before entry into the organelle (Deane et al., 2001). Taken together, Engman and colleagues proposed a pathway for ciliary membrane trafficking based on the targeted delivery model, in which specific vesicle transport is crucial (For a detailed review, seen in (Emmer et al., 2010). Briefly, the flagellar lipids and membrane proteins (e.g. calflagin) are loaded, together with one or more cilium-specific palmitoyl acyltransferases (PATs), into vesicles that are destined for the cilium. The palmitoylation may occur in the vesicle which contains the myristoylated protein and its specific PAT (Emmer, personal communication). The targeted vesicles are transported to the base of the cilium, and fuse with periciliary membrane to allow ciliary lipids and proteins to enter into the organelle. There are at least two decision points in this model. One that has been mentioned is for the vesicles, where the various destinations are decided (e.g. periciliary membrane or

plasma membrane). Several members have been implicated in this process, including the small guanosine triphosphate hydrolases (GTPases) of the Arf, Arl and Rab families (Stenmark, 2009, Gillingham and Munro, 2007). On the other hand, it is obvious that not all the proteins are loaded into the cilium-specific vesicles. A decision point for the selective load is also likely to exist. Previous studies revealed a number of dually acylated proteins localized in the flagellum. As proved in this study, the N-terminal dually acylation is not the actual flagellar targeting signal. Therefore, proteins have to be loaded into the cilium-specific vesicles in the presence of the additional flagellar signals.

6.3.4 A model for the flagellar targeting of dually acylated proteins in *T.brucei*

The crucial step of the above model is the vesicles targeting loads to the periciliary membranes at the base of cilium, where polarized exocytosis occurs. However, the periciliary membrane can take different forms in different cell types. The flagellar pocket of *T.brucei* is a unique periciliary membrane which is much larger than that of most cells, and more importantly, all endocytosis and exocytosis occurs exclusively in the flagellar pocket. In addition, the subpellicular array microtubules are too closely spaced to allow transport vesicles to access the plasma membrane (Field and Carrington, 2009). The flagellar pocket may serve as the central space for membrane protein transportation and provide access to multiple destinations. Consequently, the targeted-vesicles model is probably simplified as all vesicles are destined to the flagellar pocket. Instead of being selectively loaded into the cilium-specific vesicles, the N-terminal dual acylation proteins may be non-specifically sorted to the flagellar pocket,

and redirect to other destinations based on additional signals.

Considering the ‘kinetic bilayer trapping’ hypothesis and the existing data, I have proposed a model of dual-acylation-mediated protein trafficking that incorporates the involvement of the flagellar pocket (FP) membrane and flagellar pore complex in *T.brucei* (Fig.17). The myristoylated but not palmitoylated proteins can be transiently localized to multiple cellular membranes (except for the flagellar membrane), and rapidly palmitoylated by one of many palmitoyl transferases (PATs). There are 12 PATs in *T.brucei*, but their localization has not been experimentally determined (Emmer et al., 2009). The palmitoylation may occur at several sites (Fig.15). The fully acylated proteins are stably associated with the FP membrane, transported through either the non-vesicle-mediated or vesicle-mediated traffic systems. Additional signals are recognized and direct proteins to various destinations. In case of CALP1.3 and SKCRP1.5, these signals mediate entry into the flagellum. The model implies a role of N-terminal dual acylation of membrane-association of non-transmembrane proteins in kinetoplastids. The crucial factor is a stable association with the flagellar pocket membrane, which operates as a decision point for the subsequent targeting steps. Therefore, the role of dual lipid modification is to serve as a membrane anchor, providing a high affinity to the membrane of flagellar pocket, whereas the precise destinations are determined by additional signals.

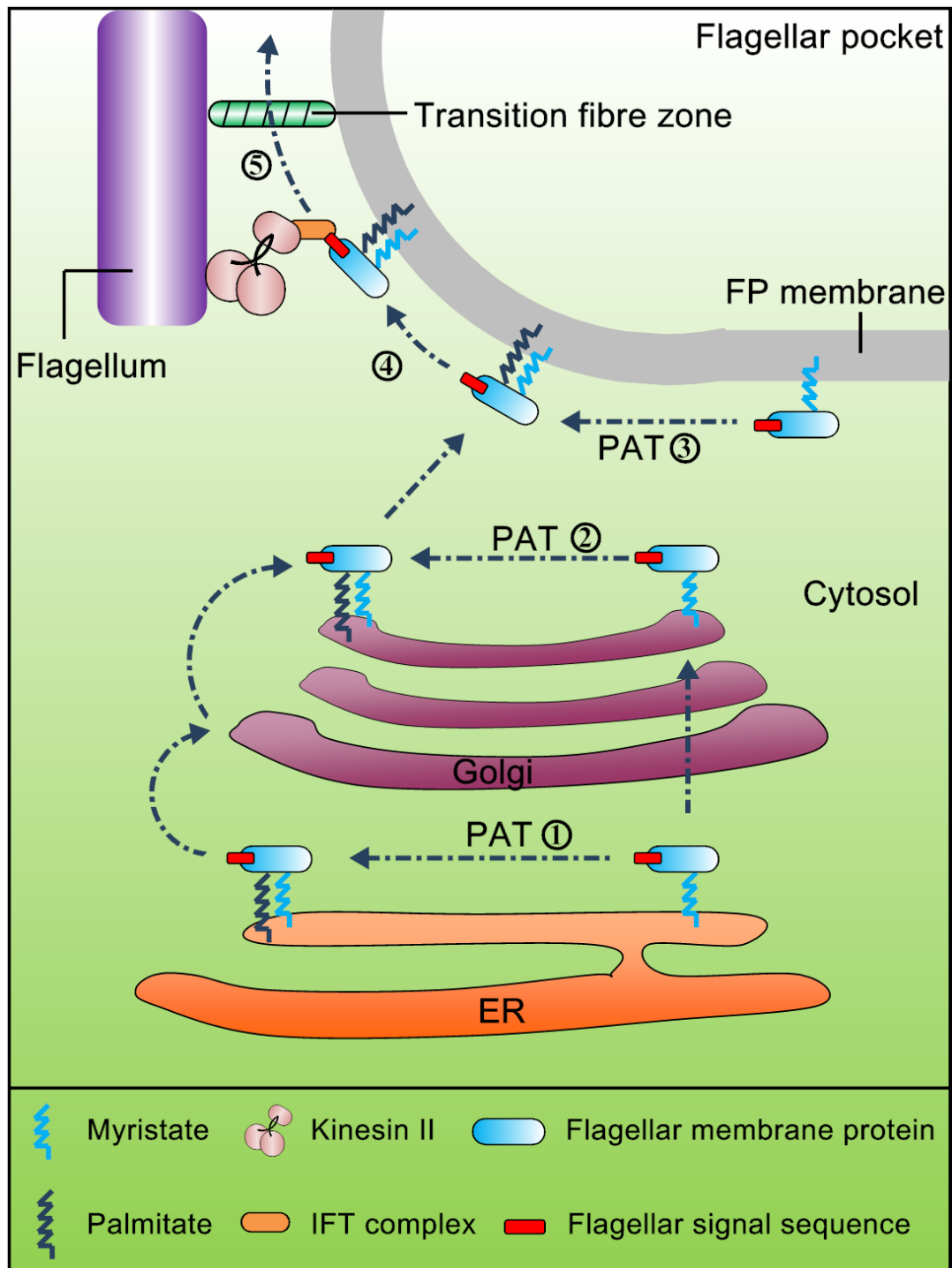


Fig.17 An acylation-mediated model of subcellular trafficking of flagellar membrane proteins.

Single myristoylated protein may transiently localize in ER membrane(1) and subsequently palmitoylated by a putative PAT. Dually acylated protein can then traffic to the Golgi and FP membrane via the vesicle-mediated secretory pathway. Alternatively, single myristoylated protein may also transiently localize in the Golgi membrane(2) or FP membrane(3) through a non-vesicular pathways and be palmitoylated. Once stably associated with the FP membrane, flagellar signals are recognized by interacting with IFT systems(4), which will then drive the ‘cargo’ passing through the transition fibre into the flagellar microenvironment(5). IFT system and the heterotrimeric kinesin II complex are crucial to the trafficking mechanisms.

6.3.5 The flagellar pocket is a highly dynamic organelle for protein trafficking

The 1.5N_GFP fusion was targeted to the cytoskeleton, indicating the first 30 amino acids of SKCRP1.5 are probably not harboring sufficient signal for its flagellar localization. However, although the signals for the precise localization were depleted, no obvious accumulation in the flagellar pocket has been observed. 1.5N_GFP fusions were effectively targeted to the cytoskeleton rather than simply accumulated in the flagellar pocket. One explanation is saturable retention in the flagellar pocket. Previous studies have shown that overexpression of the FP-specific transferrin receptor lead to an escape to the plasma membrane (Mussmann et al., 2003). In this study, overproduction of CALP1.3^{myc} resulted in the signals expanding from the tip of flagellum, to the entire flagellar membrane, and then to the plasma membrane, with a final distribution throughout the cell body. This may imply that the flagellar pocket is a highly dynamic area with limited restrictive abilities (Field and Carrington, 2009), and once the precursor have entered, it either initiates the transportation based on a recognizable signal (if any) or lets it passively distribute to the rest of the cell space.

What constitutes a ‘recognizable signal’? It has been shown that CALP1.3 N terminus is sufficient for flagellar targeting. However, when the C-terminal 35 amino acids of CALP1.3 were deleted, it was not localized to the flagellar membrane but homogenously localized in the cell body (Fig.10b). In general, trafficking of membrane and cytoskeletal proteins to a certain intracellular position may take place via a simple linear targeting peptide sequence or through a more complex 3D-structure-dependent signal patch consisting of several region of the protein (Fridberg et al., 2007). Therefore, for the CALP1.3 C-terminal deletion mutants, one possible explanation may be the

structural change due to the sequence loss: a C-terminal 35 or more amino acids deletion (up to 194aa in this study) might have caused the conformational change that inactivated the flagellar signal at the N-terminal region, resulting in protein mis-targeting.

7. Concluding remarks

The purpose of this study of calpain-like proteins in *T.brucei* was the description of basic biochemical and cell biological features of this very diverse kinetoplastid protein family in order to establish a foundation for targeted approaches aiming at functional characterization (summarized in Table 3).

Name	Size (kDa)	Expression specificity	Subcellular localization	N-terminal lipid modification
CALP1.3	82.6	Non	Flagellar tip	Dual acylation
SKCRP1.4	15.9	Non	Cell body	Single acylation *
SKCRP1.5	14.7	Non	Flagellar	Dual acylation
SKCRP1.6	13.5	PCF-specific	Plasma membrane	Dual acylation *
SKCRP1.7	14.0	Non	Plasma membrane	Dual acylation *
CALP4.2	89.8	Non	Cell body	Non
CALP6.1	113.3	Non	Cell body	Non
SKCRP7.1	12.8	Non	Cell body	Non
SKCRP7.2	13.5	PCF-specific	Cell body & Flagellar tip	Non
CALP8.1	98.4	BSF-specific	Subpellicular cytoskeleton	Dual acylation *

Table.3

Summary of the features of some calpain-like protein studied in this thesis. * indicates the N-terminal lipid modification has not been experimentally confirmed but from sequence prediction.

Precise functions of these proteins, which most likely do not even possess proteolytic activity, are not yet known. A study in *T. cruzi* epimastigotes found a correlation between experimentally induced stress (nutritional, pH, temperature) and increased expression levels of a *T. cruzi* CALPx.11 protein (Giese et al., 2008). The sequence

homologue of CALPx.11 in *T.brucei* is TbCALP1.2. Depletion of TbCALP1.2 in bloodstream cells in a systematic RNAi-based study of *T.brucei* chromosome 1 genes revealed a growth phenotype (Subramaniam et al., 2006). The same RNAi-screen showed no phenotypes for TbCALP1.1, 1.3 and 1.4. A proteomics analysis of proteins differentially expressed in benznidazol-resistant *T. cruzi* epimastigotes identified *T.cruzi* SKCRPx.1 as being upregulated (Andrade, 2008). In *Leishmania donovani* SKCRP14.1 expression is associated with drug-induced programmed cell death and the modulation of susceptibility to antimonial drugs (Vergnes et al., 2007). Depletion of the life-cycle specific proteins TbCALP4.1/CAP5.5 and CALP8.1/CAP5.5V interferes with cytokinesis and organelle positioning in procyclic and bloodstream cells, respectively (Olego-Fernandez et al., 2009). The localisation of some calpain-like proteins at the tip of the flagellum suggests possible involvement in sensory functions. Based on observations made for typical, enzymatically active calpains, but also for an emerging class of pseudoenzymes, non-enzymatic proteins with significant sequence similarities to enzymes such as kinases and proteases, we have previously hypothesized that calpain-like proteins could be involved in regulatory processes (Ersfeld et al., 2005, Pils and Schultz, 2004b, Todd et al., 2002). Although the data available in the literature and presented in this study are compatible with this view, detailed functional studies will be necessary to address this issue.

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9. Appendix

Appendix 1

Name	Vector	Primer sequences	
Primers for full length gene expression			
CALP1.3	pHD1700	For	GGCAAGCTTATGGGGTGTGGTGCGTCTTC
		Rev	CGGGGATCCCTTTTTTTTATTTTCCACAT
SKCRP1.4	pLEW111	For	GCAAGCTTAAAGATGGGTCTATTTCTGAGCAAACAA
		Rev	GCCTCGAGAAACGATGTCGCCTTGAAGGGACC
SKCRP1.5	pLEW111	For	GCAAGCTTCAAAATGGGTTGCATTCAGTCGACAGTC
		Rev	GCCTCGAGTAGACGATATTGTTCTTCAGATCCTGC
SKCRP1.6	pHD1700	For	GGCAAGCTTATGGGTTGTGGTGGCTCAAA
		Rev	GGCGGATCCCTATTGCCTGTCTCCATCAGT'
SKCRP1.7	pHD1700	For	GGCAAGCTTATGGGTTGCGGTGGCTCAAC
		Rev	GGCGGATCCTTTCCTGCCAGATCCCCTGC
CALP4.2	pLEW111	For	GCAAGCTTCGAAATGACGAGCAGCGGTGGTCTTCT
		Rev	GCCTCGAGCTCACCCAAATCGAACTCTTTCAGCC
CALP6.1	pLEW111	For	GCAAGCTTTGCAATGGTTGTGAGTCTTCCGGTTA
		Rev	GCCTCGAGAACACTCGACAACACTCTAACAGAAAAA
SKCRP7.1	pHD1700	For	CGAAGCTTATGTCCGATATCACGTACGAGAAC
		Rev	GCGGATCCTGACTGAGGAATAGGATTGGCTTCA
SKCRP7.2	pHD1700	For	GCAAGCTTATGTCCGATATCACGTACGAGAAC
		Rev	GCGGATCCGGTGGGAACGGATTTCCTACTG
Primers for mutagenesis study			
CALP1.3 Δ3	pHD1700	For	AAGCTTATGGGGTGTGGTGCGTCTTC
		Rev	CGGGGATCCATTTTCCACATACCAGTCGC
CALP1.3 Δ14	pHD1700	For	AAGCTTATGGGGTGTGGTGCGTCTTC
		Rev	CGGGGATCCTACTATCACAGTGCTGTTAT
CALP1.3 Δ35	pHD1700	For	AAGCTTATGGGGTGTGGTGCGTCTTC
		Rev	CGGGGATCCTGGGACGCTGAAGAACTCTG
CALP1.3 Δ74	pHD1700	For	AAGCTTATGGGGTGTGGTGCGTCTTC
		Rev	CTCGAGTCCTGTAATGATGCGTGGCA
CALP1.3 Δ193	pHD1700	For	AAGCTTATGGGGTGTGGTGCGTCTTC
		Rev	CTCGAGGCTCCTCGGGTAGCGGAAAA
CALP1.3 ΔG2	pHD1700	For	GGCAAGCTTATGGTGGGTGCGTCTTCTACTCA
		Rev	GGCGGATCCCTTTTTTTTATTTTCCACAT
CALP1.3 ΔC3	pHD1700	For	GGCAAGCTTATGGGGGGTGCCTTCTACTCA
		Rev	GGCGGATCCCTTTTTTTTATTTTCCACAT
CALP1.3 ΔGC	pHD1700	For	GGCGGGCCCATGGGTGCGTCTTCTACTCA
		Rev	GGCGGATCCCTTTTTTTTATTTTCCACAT

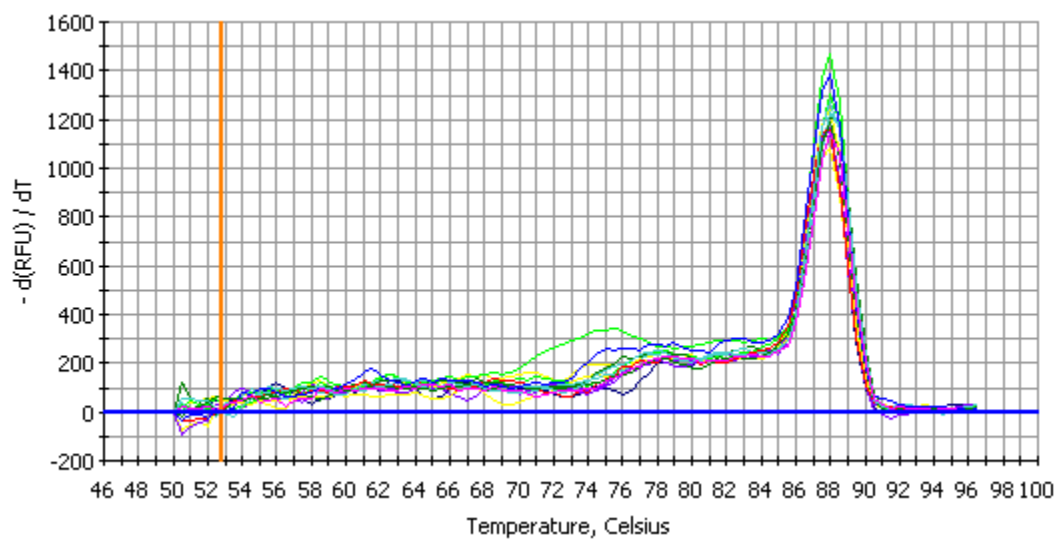
Name	Vector	Primer sequences	
Primers for mutagenesis study(continued)			
SKCRP1.5 ΔG2	pHD1700	For	GGCAAGCTTATGTGCATTCAGTCGACAGT
		Rev	GGCGGATCCTAGACGATATTGTTCTTCAG
SKCRP1.5 ΔC3	pHD1700	For	GGCAAGCTTATGTGCATTCAGTCGACAGT
		Rev	GGCGGATCCTAGACGATATTGTTCTTCAG
SKCRP1.5 ΔGC	pHD1700	For	GGCAAGCTTATGATTCAGTCGACAGTCAG
		Rev	GGCGGATCCTAGACGATATTGTTCTTCAG
Primers for GFP hybrid protein study			
1.3WT_GFP	pHD-GFP	For	GGCAAGCTTATGGGGTGTGGTGCCTCTTC
		Rev	CGGACGCGTCTTTTTTTTATTTTCCACAT
1.3N_GFP	pHD-GFP	For	GGCAAGCTTATGGGGTGTGGTGCCTCTTC
		Rev	CGGACGCGTGGCTGCAATTTGCTTGCGCA
1.5WT_GFP	pHD-GFP	For	GGCAAGCTTATGGGTTGCATTCAGTCGAC
		Rev	GGCACGCGTTAGACGATATTGTTCTTCAG
1.5N_GFP	pHD-GFP	For	GGCAAGCTTATGGGTTGCATTCAGTCGAC
		Rev	CGGACGCGTAAGTCCATTAAACATGCTGT

Appendix 2

Name	Gene ID	T _m (°C)	kDa	Sequence	
PFR-A	Tb927.8.4990	55	69.6	For	5' CCGCAGCAAGATGGTGGAG 3'
				Rev	5' GCGGTACTGCTGGCTCTGG 3'
CALP1.1	Tb927.1.2100	55	126.2	For	5' CGCTCCCTTGTCTGGGAA 3'
				Rev	5' GTAAGGAGACCCACGCTT 3'
CALP1.2	Tb927.1.2110	55	75.5	For	5' TCGACGCGGACCATCCCA 3'
				Rev	5' GGACAGCTCAATAGAGTTACCA 3'
CALP1.3	Tb927.1.2120	55	82.6	For	5' TGGAGGCGAAATTCCAAGT 3'
				Rev	5' TCCACATACCAGTCGCCC 3'
SKCRP1.4	Tb927.1.2150	55	15.9	For	5' CGCAACGTTTCGTTAAGGGAG 3'
				Rev	5' CGATGTGCCTTGAAGGG 3'
SKCRP1.5	Tb927.1.2160	51	14.7	For	5' GCCTTCCGAATTGATGAGC 3'
				Rev	5' GTTCTTCAGATCCTGCGGTAC 3'
SKCRP1.6	Tb927.1.2230	51	13.5	For	5' AACACGCGCTGGTCGCTG 3'
				Rev	5' ATTGCCTGTCTCCATCAG 3'
SKCRP1.7	Tb927.1.2260	51	14.0	For	5' AAGCACTGGTCGCTGGGC 3'
				Rev	5' CCATTCACAGTGCCTTCA 3'
CALP4.1	Tb927.4.3950	55	94.6	For	5' CATCGGATGCAGCCACCC 3'
				Rev	5' ATGAACCCGAGCCGGCAA 3'
CALP4.2	Tb927.4.3940	55	89.8	For	5' GGATGTGGAGATCCCCGG 3'
				Rev	5' ATCATGGATGCGCGGAAT 3'
SKCRP5.1	Tb927.5.2190	54	48.8	For	5' AAATCAAAGGTACGCGGTCT 3'
				Rev	5' CGTTTACATCCCCAGCAAC 3'
CALP6.1	Tb927.6.3310	54	113.3	For	5' TCAAGGGGAAAATACCAGC 3'
				Rev	5' GCAGACAAGGAATAACCAAAA 3'
SKCRP7.1	Tb927.7.4060	55	12.8	For	5' CATGGTGGTGAAAGTTGC 3'
				Rev	5' ATGACTGAGGAATAGGATTGGC 3'
SKCRP7.2	Tb927.7.4070	55	13.5	For	5' GACACTGCTACTGGCGAA 3'
				Rev	5' GGTGGGAACGGATTTCCAC 3'
CALP8.1	Tb927.8.8330	55	98.4	For	5' CGAGTCACCGCAGCAATCA 3'
				Rev	5' GCAAATCTTCCCAGTCCATCAT 3'
CALP9.1	Tb09.160.5550	54	95.4	For	5' AAGCGTGGTTTACGCATC 3'
				Rev	5' GAGCAACGAACACCGAGA 3'
CALP10.1	Tb10.389.0470	54	75.5	For	5' ATGAAGCCTCAGGAATCATAT 3'
				Rev	5' GACACGCACTCAATGGTAAA 3'
CALP10.2	Tb10.70.5950	56	178.1	For	5' ACCCGTTGGAAAAGAAGC 3'
				Rev	5' CACCATTTCATCGCCTTC 3'
SKCRP10.3	Tb10.70.5060	54	54.4	For	5' AGGTGGCACCCCTTTCTA 3'
				Rev	5' CAAGGAGAATCCGCTGTGA 3'
CALP11.3	Tb11.02.2240	57	117.2	For	5' TGAAGGCGGAGGTGTTGT 3'
				Rev	5' ACCATAGTGCGGGAAGCT 3'

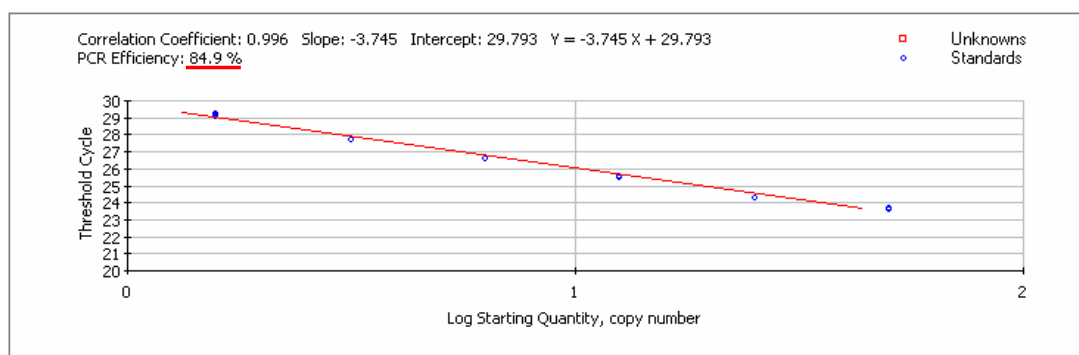
Appendix 3

Melt Curve Graph for PFR-A

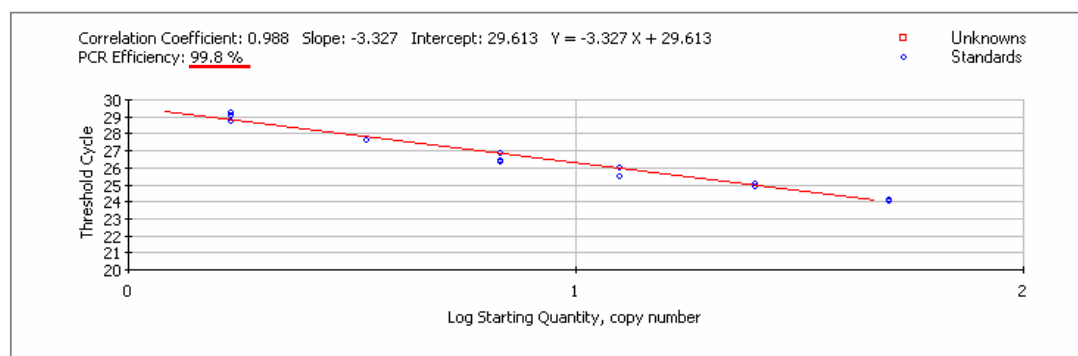


Appendix 4

Efficiency Curve of CALP1.3



Efficiency Curve of PFR-A



Appendix 5

SKCRP1.6&1.7

1	MGCGGS	X	T	S	T	V	E	F	I	N	G	P	T	V	Q	G	D	E	I	V	K	G	F	N	E	G	N	G	L	L	F	R	I	V	K	T	R	A	G	R	W	A	Y	N	D	T	L	D	Y	D	M	H	V	K	V	T	F	S	E	D	C	R	I	1.6
1	MGCGGS	-	T	S	V	T	F	I	N	G	R	P	T	V	Q	G	D	E	I	A	K	G	F	N	E	G	N	G	L	L	F	R	I	V	N	S	S	T	G	R	W	A	Y	N	D	T	L	D	Y	D	M	H	V	K	V	T	F	S	E	D	C	K	I	1.7
71	KALG	Q	T	R	L	E	K	L	E	S	G	E	S	V	A	T	V	V	V	K	P	C	A	T	E	L	F	I	E	G	H	V	N	G	Y	K	A	K	M	D	A	I	P	I	T	D	G	D	R	Q	1.6													
70	KALG	K	A	K	L	E	R	L	S	G	E	S	V	V	T	V	V	V	K	P	C	A	T	E	L	F	I	E	G	T	V	N	G	Y	K	A	K	M	D	A	I	P	I	T	D	G	D	R	R	R	G	S	G	R	K	1.7								

SKCRP7.1&7.2

1	MS	D	I	T	Y	E	N	G	S	P	T	Y	T	G	N	T	V	L	K	C	F	R	E	N	G	N	L	L	F	R	I	V	N	D	E	E	K	K	W	A	F	Y	N	D	T	K	G	Y	N	M	V	V	K	V	A	F	G	K	D	S	T	V	Q	P	L	G	N	T	7.1																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																													
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